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The effects of giving rabbits a minimal lethal dose (0.22 ug/kg) of ricin on their hearts and coronary arteries were investigated. Ricin did not alter the time to peak tension of the papillary muscle and its contraction duration, or frequency responses. The EC_{50} for force development of the papillary muscle in the presence of Bay K 8644 was increased by ricin. Ricin did not alter the resting membrane potential, action potential amplitude, action potential duration, overshoot potential, the effective refractory period, or the maximal dv/dt of Phase 0 of papillary muscle but increased monoamine oxidase activity of papillary muscle. The number and affinity of beta receptors and oxygen consumption in the heart were not altered. Ricin did not alter heart rate, P-R

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interval, or QRS duration of the ECG, but decreaseed compliance of the isolated heart. Neither isoproterenol- nor phenylephrine-stimulated left ventricular developed pressure, +dp/dt or -dp/dt were changed. Ricin increased the EC_{50} for contractions of coronary artery to serotonin and histamine with or without endothelium present. It decreased the EC_{50} for relaxations of the coronary artery to norepinephrine, but not acetylcholine. Thus, ricin alters coronary artery function, and reduces diastolic and systolic function of the heart.

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Casey Robinson
PI - Signature

January 20, 1994
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I. INTRODUCTION

A. Nature of the Problem

Ricin is a threat agent. Understanding its actions and effects is important in designing therapy for and prophylaxis against ricin intoxication. Among the poorly understood aspects of its effects are those on the cardiovascular system.

B. Background of Previous Work

1. General

Ricin is a toxic lectin from the castor bean (*Ricinus communis*) which exists in slightly different forms in seeds of different origin. Ricin D is isolated from large castor beans which originate in Thailand whereas ricin E comes from small castor beans cultivated in Japan (Hatakeyama *et al.*, 1989). These differ in amino acid sequence as well as toxicity. Ricin consists of two different polypeptide chains linked together by a single disulfide bond. The B chain binds the toxin to cell surface receptors containing terminal galactose residues (Nicolson and Blaustein, 1972). This appears to be an obligatory step in the intoxication of the cell (Olsnes *et al.*, 1974; Olsnes *et al.*, 1976). In the entry process the disulfide bond is broken and the free A chain exhibits the toxic action on the cell by inhibiting protein synthesis (Olsnes *et al.*, 1976). The extreme toxicity of ricin is due to the fact that the liberated A chain is an enzyme and a single A chain in the cell may be sufficient to kill the cell (Eiklid *et al.*, 1980). Even though ricin is toxic to all nucleated mammalian cells tested (Olsnes and Pihl, 1982), it appears that it is more toxic to certain cells (Fodstad and Pihl,, 1978). Ricin and related proteins have been reviewed by Olsnes and Pihl (1976; 1982).

2. Chemistry of Ricin

The primary structure of ricin D has been determined by Funatsu *et al.* (1978; 1979). The sequence of the B chain was redetermined and revised in 1985 by Araki and Funatsu. There are 4 internal disulfide bridges in the B chain, but none in the A chain. There are no reactive sulfhydryl groups on the intact toxin. However, in the presence of sodium dodecyl sulfate (Cawley and Houston, 1979) and 6 M guanidine chloride (Yoshitake *et al.*, 1978) a buried sulfhydryl group can be uncovered in position 171 of the A chain. The neutral A chain in native ricin can be observed as a doublet when analyzed by polyacrylamide gel electrophoresis. These have been designated as an A_1 component (64%) with a molecular weight of 30,000 and an A_2 component (36%) with a molecular weight of 32,000. This higher molecular weight component contains an additional second mannose-rich oligosaccharide (Foxwell *et al.*, 1985). The A chain has 265 amino acid residues with sequences

consisting of both hydrophilic and hydrophobic sections, and has a helical content estimated at 0.3% (Funatsu *et al.*, 1973). The acidic B chain has 262 amino acid residues with a molecular weight of 31,557 (Araki and Funatsu, 1987) and has a helical content of 10% (Funatsu *et al.*, 1979). The B chain has four internal disulfide bridges linking cysteine residues at positions 20 to 39, 63 to 80, 151 to 164 and 190 to 207 (Araki and Funatsu, 1985). Even though weak interactions would hold the chains together, the presence of the intact disulfide bridge linking the two appears to be necessary for ricin's toxic effect. However this link must be reversible in order to release the free A chain into the cell. When a covalent interchain crossing of ricin is made with N,N'-*O*-phenylenedimaleimide, a non-toxic product results. Similar treatment of the free A chain did not alter toxicity (Oda and Funatsu, 1979). The A chain of ricin D and ricin E is identical. The amino acid sequence for the B chain of ricin E was determined by Araki and Funatsu (1987). The B chain of ricin E contains 262 amino acid residues and is composed of the N-terminal half of ricin D and the C-terminal half of Ricinus communis agglutinin, another toxic lectin found in the castor bean (Araki *et al.*, 1986).

The amount of carbohydrate in the structures is still under debate but it is clear that most of the carbohydrate in the molecule is associated with the B chain. Funatsu *et al.* (1971) and Nanno *et al.* (1975) found one oligosaccharide chain on the A chain consisting of $(GlcNAc)_2(Man)_4$. Whereas Foxwell *et al.* (1985) found one oligosaccharide chain on the A_1 component of the composition $(GlcNAc)_2(Xyl)_1(Fuc)_1(Man)_{3-4}$ and evidence for another oligosaccharide chain on the A_2 chain consisting of only GlcNAc and Man. Funatsu *et al.* (1971) found two chains on the B chain consisting of $(GlcNAc)_2(Man)_6$ and $(GlcNAc)_2(Man)_7$. These oligosaccharide chains are each attached to asparagine residues. In addition ricin binds to concanavalin A indicating that the mannose residues are exposed. Crystallization studies of ricin have been carried out. These show only one ricin molecule per asymmetric unit. A low resolution study (4 Å) showed a bilobal structure for ricin B chain with each domain being able to bind a galactose residue (Villafranca and Robertus, 1981).

Ricin can be purified by affinity chromatography on Sepharose 4B. This matrix which contains ß-galactose residues, binds the ricin toxin as well as ricinus agglutinins.

These can be separated by N-acetyl-galactosamine which only elutes the ricin toxin; the agglutinin requires elution by galactose (Nicolson *et al.*, 1974).

3. Mechanism of Action

It has long been known that ricin interferes with protein synthesis. Olsnes *et al.* (1974) concluded that ricin inactivated the 60S ribosomal subunit making it unable to interact with the EF2 elongation factor. Endo

et al. (1987) found that the target of the toxin was the rRNA as opposed to the ribosomal proteins in the 60S subunit. Their evidence showed that the 28S rRNA of the 60S subunit had reduced mobility in electrophoretic gel after treatment with ricin. This change in migration disappeared when the samples were analyzed by gel electrophoresis in the presence of urea. These data led to the conclusion that the change in mobility was due to a conformational or chemical modification rather than a change in length of the fragment. Determination of the nucleotide sequence by enzymatic digestion revealed a missing adenine residue at position 4324 in the ricin treated ribosomes. This missing adenine rendered the fragment susceptible to digestion by nucleases. That the nucleotide sequence around position 4324 is part of a highly conserved sequence between species and the toxin is active only in eukaryotes, indicates that the toxin must recognize a specific conformation and not a nucleotide sequence. In 1987, Endo and Tsurugi quantitated the amount of adenine liberated per mole of ribosomes treated with ricin. They discovered that almost 1 mole of adenine (0.78 to 0.84 mole) was liberated per mole of ribosomes treated. They also treated ribosomes with ricin in the presence of [P^{32}]phosphate. Less than 1 mole of the phosphate was incorporated per 100 moles of the modified ribosomes. They concluded that the ricin A chain removes the adenine residue by hydrolysis and does not act as a phosphorolytic enzyme. They further concluded that ricin A chain has RNA *N*-glycosidase activity cleaving the *N*-glycosidic bond of a single adenine residue (A^{4324}) of the 28S rRNA in a hydrolytic fashion, leaving the phosphoribose backbone intact. This removal does not require any cofactors since ricin will inactivate isolated ribosomes (Olsnes *et al.*, 1974). Olsnes *et al.* (1975) showed in kinetic experiments that ricin A chains in simple buffer solution inactivated salt-washed ribosomes at a rate of about 1500 ribosomes per minute per ricin A chain. The Q_{10} was about 1.8 and the K_m about 1-2 X 10^{-7}M. The inactivation of ribosomes could be halted at any time by adding specific anti-A chain antibodies. Several plant toxins have similar structure and action to ricin. Two of these, abrin and modeccin also remove adenine from A^{4324} (Endo *et al.*, 1987).

4. Steps in Ricin Toxicity

The B chain is responsible for the binding of the toxin to the cell surface. The chain combines with galactose moieties on the cell surface. Baenziger and Fiete (1979) studied the specificity of this binding by measuring the association constant (K_a) of ricin with a series of glycopeptides of known composition. They concluded that ß-1,4-linked galactose residues are primarily responsible for binding. The oligosaccharides which bind ricin can be found on a variety of glycoproteins and glycolipids. Therefore the toxin can bind to several different molecular species. Binding studies have shown that HeLa cells possess 3 X 10^7 binding sites for ricin, even though studies have shown that a single toxin molecule within the cytosol is sufficient to ensure cell death (Eiklid *et al.*, 1980). Hatakeyama *et al.* (1989; 1990) reported two binding sites on the B chain, a high affinity binding site (HA) and a low

affinity site (LA). The ethoxyformylation of histidine residues in ricin E abolishes the saccharide binding capability of the HA site, indicating that one of the three histidine residues must be present at the HA site.

After combining at the cell surface the B chain facilitates the entry of the A chain into the cell where it interacts with the 60S ribosomal subunit and interferes with protein synthesis (Olsnes *et al.*, 1974). The A chain is transported into the cell complexed to the receptor in a process called receptor-mediated endocytosis. It is then stored in endosomes until it is released into the cytosol (Olsnes and Sandvig, 1983). According to work done by Moya *et al.* (1985), ricin entry into the cell is not dependent on coated pits. After hypotonic shock and incubation in K^+-free media, cells will arrest their clathrin coated pit formation (Larkin *et al.*, 1983). After this treatment the ricin still retained the original amount of cell toxicity. Sandvig and Olsnes (1982) tried several treatments in an attempt to alter the entry of ricin into the cell, in order to better understand the process. They found that lowering the pH below neutral decreases the toxicity of ricin and that at pH 6.0 the toxin was unable to inhibit protein synthesis. Also a pH of 8.0 increased the cell's sensitivity to ricin. Their studies showed an increased Ca^{2+} uptake at this higher pH. Their further studies indicate that this pH effect is not due to reduced endocytosis or reduced binding to the cell surface. Since endocytosis has been reported to be an energy dependent process (Silverstein *et al.*, 1977), Sandvig and Olsnes (1982) investigated the effect of metabolic inhibitors on the toxicity of ricin. A combination of 2-deoxyglucose, an inhibitor of glycolysis, and sodium azide, an inhibitor of oxidative phosphorylation, provided strong protection against the toxin. 2-deoxyglucose by itself will not inhibit endocytosis, but does provide some protection against the toxin. This may be due to an interference with the movement of material between different membrane compartments. Calcium in the media and its influx into the cell are not required for entry of ricin into the cell.

A deprivation of Ca^{2+} in the media provided only partial protection against ricin intoxication. Verapamil, a calcium channel antagonist, offered moderate protection against intoxication. This partial protection by Ca^{2+} deprivation and a calcium channel blocker along with the increased toxicity at higher pH where Ca^{2+} influx is increased seems to indicate that ricin is internalized by two mechanisms, one of which is Ca^{2+} dependent and another which is Ca^{2+} independent (Sandvig and Olsnes, 1982). Sodium deprivation afforded no protection. The presence of trivalent cations of the lanthanide series provided strong protection against intoxication. These cations markedly inhibit the uptake of Ca^{2+} by the cells. However, Fe^{3+} which does not affect calcium flux, also provides good protection against ricin intoxication, so the protection afforded by the lanthanides may come from another mechanism and not from their effects on calcium flux. These treatments do not affect the ability of the ricin A-chain to inactivate ribosomes in cell-free systems. Therefore, all of these protective effects are due to the inability of the toxin to enter the cytosol and gain access to the ribosomes. Internalization of the ricin toxin

is a very slow process with only about 8% of cell-bound toxin internalized each 10 minutes at 37°C in Hep_2 cells with functional coated pits (Moya *et al.*, 1985).

After endocytosis, ricin is not easily degraded and only small amounts appear to be accumulated in lysosomes. Two hours after internalization, about 90% of internalized ricin remained intact (Sandvig *et al.*, 1978). Later, part of the endocytosed ricin was released back into the medium, presumably by recycling of the receptor-toxin complex back to the cell surface (Sandvig and Olsnes, 1979). It had been suggested that ricin enters the cytosol by the rupture of endocytic vesicles (Nicholson, 1974; Nicholson *et al.*, 1975). However, in the light of the work by Sandvig and Olsnes (1982) this unspecific vesicle rupture does not seem likely. They showed that low pH and absence of Ca^{2+} protected well against the toxicity of ricin and related toxins, abrin and modeccin, but not against diphtheria toxin in spite of the fact that all were endocytosed equally under these conditions. The transfer of endocytosed ricin to the Golgi complex appears to be necessary for intoxication (Sandvig *et al.*, 1986). Studies involving the labeling of ricin with a horseradish peroxidase (van Deurs *et al.*, 1986) found that ricin was routed through the vacuolar and tubulo-vesicular portions of the endosomal system on its way to the Golgi complex. With immunogold labeling, Hansen *et al.* (1989) demonstrated the localization of ricin in Golgi stacks and associated *trans*-Golgi network. Evidence now shows that ricin is translocated to the cytoplasm from the *trans*-Golgi network. Van Deurs *et al.* (1990) present a scheme for intracellular routing and sorting of ricin based on current knowledge and speculation. Ricin, bound to membrane glycoproteins and glycolipids, is internalized by both uncoated and coated pits and vesicles to reach endosomes. From the endosomes it may be rapidly recycled to the cell surface, transferred to lysosomes where it is slowly degraded or delivered to the *trans*-Golgi network. From the *trans*-Golgi network it can be routed back to the cell surface or translocated into the cytosol where it can inhibit protein synthesis.

5. Toxicity

a. Cell Culture

Toxicity of ricin in cell culture was first shown by Lin *et al.* (1970, 1971). The earliest toxic effect of the toxin observed is protein synthesis inhibition. Later DNA and then RNA synthesis is inhibited. There is no effect on energy metabolism or oxidative phosphorylation. Data show that protein synthesis inhibition is primarily responsible for cell death. Ricin is toxic in tissue culture in concentrations of about 1 ng/ml. A lag time is apparent after the addition of ricin to a cell culture. This lag time can be reduced by increasing the concentration of the toxin, but the lag time is always more than 20 to 30 minutes even at high concentrations (Refsnes *et al.*, 1974; Olsnes *et al.*, 1976). The synthesis of proteins may be inhibited to different extents. For example in a myeloma cell line, the

synthesis of a myeloma protein (IgA) was more rapidly inhibited than bulk protein synthesis (Ko and Kaji, 1975). Different cell lines differ in sensitivity to ricin, but this sensitivity does not correlate well between animals and cell culture. Ricin D and ricin E are equally toxic in mice, however in cell culture ricin D is much more toxic than ricin E (Koga *et al.*, 1979). After intoxication the cell undergoes early morphological changes. The cell surface becomes irregular, but the surface membrane remains functional even after all protein synthesis ceases. This was demonstrated by the cells continuing ability to exclude trypan blue (Nicholson *et al.*, 1975; Lin *et al.*, 1970).

b. Animals

Ricin is especially toxic after parenteral administration, but even after oral administration it still exhibits toxicity. The toxicity of ricin varies among species with the guinea pig more sensitive on a weight basis than the mouse and the horse being the most sensitive animal of those tested (Ehrlich, 1957; Balint, 1974). Fodstad *et al.* (1976) determined an approximate LD_{50} in B6D2 mice of 55 to 65 ng/mouse (mice weighing 22-26 g). The distribution of ricin was determined by radiolabeling. The greatest amount of toxin was found in the spleen and liver with kidney and blood showing a lower level. Godal *et al.* (1984) did distribution studies with radiolabeled ricin and confirmed the high levels in the spleen and liver, but also found high levels in the bone marrow and adrenal cortex. No activity was found in the brain. They found that the sensitivity to ricin differs even between strains of the same species. These investigators felt that a minimum lethal dose (MLD) was a more meaningful parameter than an LD_{50}. The MLD in mice varied from 1.95 µg/kg for DBA mice to 2.40 µg/kg for B6D2 mice. Fodstad *et al.* (1979) found the MLD of ricin in rats, guinea pigs, rabbits and dogs to be 0.35-0.5, 0.40-0.50, 0.03-0.06, and 1.6-1.75 µg/kg respectively. Clinical symptoms of acute intoxication included loss of appetite and body weight, slight fever, edema in the extremities and ascites. Pathological findings included enlarged and congested spleen along with the reticuloendothelial cells of the liver and spleen showing increased phagocytic activity. Hematologic parameters were altered with a decrease in hematocrit and thrombocytes and an increase in leukocytes. However bone marrow examinations in dogs revealed no clear abnormality in myelopoiesis. After sub-lethal doses of ricin the animals recovered completely.

Leek *et al.* (1989) investigated the intestinal pathology following an intramuscular dose of ricin in rats. The severity of the cellular infiltration found was similar to that found in a local immune response triggered by an orally administered toxin. Griffiths *et al.* (1987) found large scale disruption in lymphoid tissue with an apoptotic type of cell death after an intramuscular dose of ricin.

The mannose-terminal oligosaccharide of the A chain acts as a ligand for the mannose receptor in macrophages *in vitro*, leading to

intoxication of the cells (Simmons *et al.*, 1986; Skilleter and Foxwell, 1986). This allows the toxin to be removed from the blood by the reticuloendothelial system and accounts for the high levels of the toxin found in the liver by Fodstad *et al.* (1976) and the severe damage which occurs in the hepatic sinusoids as reported by Derenzini *et al.* (1987).

Retrograde transport of ricin in neurons has been demonstrated by Harper *et al.* (1980). Their experiments confirmed a retrograde transport of ricin from the submandibular gland of rats to neuronal bodies in the superior cervical ganglia. Morphologic changes in the neurons support the observations from biochemical studies that ricin interferes with ribosomal function and protein synthesis. A small number of the neurons are destroyed, but no phagocytosis occurs with the capsular cells remaining intact around empty spaces. In 1982, Wiley *et al.* confirmed this retrograde transport by dipping a transected nerve into a ricin solution and then determining neuronal cell changes after 12 to 52 days. They found cell damage limited to those neurons which projected into the application site of the nerve. This damage included the disappearance of virtually all Nissl substance, pycnotic nuclei and a glial reaction. Helke *et al.* (1985) reported that the retrograde transport of ricin applies only to those nerves projecting into the periphery or located in the periphery, and not to those neurons residing entirely within the CNS.

c. Man

In man, the signs and symptoms of ricin intoxication vary greatly according to the dose of toxin administered. As is always the case, the symptoms appear only after a latent period of 8 to 10 hours. After reviewing about 700 case histories, Balint (1974) observed the following signs and symptoms. Nausea, headache, general malaise, somnolence, loss of consciousness, convulsions, bloody diarrhea with tenesmus, dehydration, thirst, cyanosis, tachycardia, fall in blood pressure, changes in electrocardiogram, asthmatic symptoms, exanthema, liver necrosis, nephritis, proteinuria, rise in excretion of non-protein nitrogen, conjunctivitis, optic nerve lesion, mydriasis, leukocytosis and changes in biological data. At post mortem the main changes noticed were bleeding in the serous membranes, hemorrhage in the stomach and intestines, degenerative changes in the heart as well as liver and kidneys, infiltrations of the lymph nodes and changes in the spleen, especially in its lymphoid elements. Specific references for each case are listed in the 1974 Balint paper.

In cases where a sub-lethal dose was administered the patient recovers with no lasting side effects (Crompton and Gall, 1980).

6. Studies on Ricin's Effects on Cardiovascular Responses and on Isolated Blood Vessels.

Appropriate doses of ricin were determined prior to determining ricin's effects in rabbits. The i.v. 48 hour (and 7 day) LD_{50} and the

minimum lethal dose (MLD) of ricin in male New Zealand White rabbits were determined by the Up and Down procedure (Dixon, 1965). A MLD and a toxic sub-lethal dose (TSD) lowered blood pressure after a 12 hour or greater lag period, but only the MLD did so significantly ($P < 0.05$) (Christiansen *et al.*, 1994a). Heart rate was increased when blood pressure was reduced, which seems to be a reflex effect, but the ECG was not altered.

Abnormal laboratory values correlated well with histological findings (Christiansen *et al.*, 1991). Serum CPK, SGPT, LDH, and cholesterol concentrations were higher and serum calcium concentrations were lower in rabbits given ricin. Rabbits that died earliest (approximately 22 hours after ricin) had marked pulmonary damage, while those that died later (36-48 hours after ricin) showed much more heart and liver damage.

Ricin increased total blood flow to most organs (Zhang *et al.*, 1992). Exceptions were the brain and lungs, where the MLD markedly reduced blood flow.

Ricin administration decreased the sensitivity of the central ear artery to norepinephrine (NE) (i.e., increased the EC_{50}) (Christiansen *et al.*, 1991b). Ricin increased methacholine (endothelium-dependent) relaxations of aorta rings, but did not alter those to papaverine.

Ricin did not alter the activity of monoamine oxidase or catechol-*O*-methyltransferase, which metabolize NE (Christiansen *et al.*, 1993a).

Ricin in some studies increased the amount of NE released by nerve stimulation, but did not alter NE reuptake by the neuronal membrane (Christiansen *et al.*, 1993b). Ricin did not alter basal calcium uptake by the aorta, but depressed stimulated calcium uptake in some studies (Hsu *et al.*, 1993). Ricin did not alter basal calcium efflux from the aorta, but increased stimulated calcium efflux.

Thus administration of a MLD or TSD ricin markedly alters blood flow distribution, reduces blood pressure, and affects several components of the vascular neuroeffector system.

C. Purposes of the Present Work

The purposes of this study are to determine the effects of ricin on the function of the heart and of the coronary arteries.

A better understanding of the cardiac effects of ricin may allow better therapy of ricin intoxication.

D. Methods of Approach

Ricin will be given to rabbits in a minimum lethal dose (MLD) and

48 hours later its effects on the heart and coronary arteries will be determined.

On coronary arteries, their abilities to contract and relax both with endothelium present and endothelium removed will be determined.

On the heart, the effects of ricin on cardiac function will be determined. Parameters measured will include: heart rate, time to maximal elastance, duration index, myocardial oxygen consumption, intraventricular pressure and volume, and effects of alpha- and beta-adrenoceptor stimulation.

Beta adrenergic receptor binding will also be determined to understand the effects of ricin on beta receptors.

The effects of ricin on electrophysiology of papillary muscle will be determined.

Effects of ricin on monoamine oxidase (MAO), catechol-*O*-methyltransferase (COMT), cyclic AMP, cyclic GMP, CK isozymes, and inositide hydrolysis will also be determined.

Effects of ricin on the action potential of the papillary muscle will be measured.

The effects of ricin on calcium uptake and release will be studied in papillary muscle, mitochondria and microsomes. Effects on basal intracellular calcium concentrations in isolated myocytes will be determined using Fura 2.

II. EXPERIMENTAL METHODS

A. Treatment of Rabbits

New Zealand White male rabbits (1.5-2.5kg), were injected with a MLD (0.22 μg/kg) of ricin or sham injected, into a marginal ear vein and sacrificed with an overdose of sodium pentobarbital (45mg/kg) or ether anesthesia 48hrs later.

B. Preparation of Rabbit Coronary Artery Rings

The heart was removed and placed into cold (10-15°C) Krebs solution of the following composition (mM): 118.5 NaCl, 4.7 KCl, 1.2 MgCl, 23.8 $NaHCO_3$, 1.2 KH_2PO_4, 11 dextrose, 2.5 $CaCl_2$, 0.01 EDTA aerated with 95% O_2/5% CO_2, pH 7.4. The left ventricular circumflex coronary artery was carefully dissected out and cleaned, while continuously perfusing with cold Krebs solution. The artery was cut into ring segments each 3 mm wide. Endothelium removal was attained by gently rubbing the inner surface with a 0.3 mm diameter stainless steel insect pin. Ring segments were mounted onto two triangular tungsten wire supports (0.006") and suspended in an isolated tissue bath containing 4 ml Krebs solution and maintained at 38°C. A resting tension of 0.6 g was applied to the coronary artery rings. The artery rings were equilibrated for 1.5 hr during which the bathing fluid was changed every 15 min. They were then exposed to 55 mM KCl for 4 min twice at 15 min intervals. Tension was measured using Metrigram isometric force transducers (Model 797159-1, Gould Inc., Cleveland OH) and recorded by a Gould RS 3800 Recorder.

C. Measurement of Contractions of Rabbit Coronary Artery Rings

After equilibration, contractions to 10^{-9} to 10^{-5} M serotonin (5-HT) and 3 X 10^{-7} to 3 X 10^{-4} M histamine were measured. The agents were added to the tissue bath in a cumulative fashion in half-log increments. Each ring segment was allowed to contract maximally to each concentration. The contraction to each concentration was reported as absolute tension. Between the two experiments, artery rings were re-equilibrated for at least 30 min with 5-min exposure to 55 mM KCl at 15 min intervals.

D. Measurement of Relaxations of Rabbit Coronary Artery Rings

Artery rings contracted to several different agonists, but those to the selective histamine receptor agonist, 2-(2-aminoethyl)pyridine (AEP) were well-maintained and were used to provide the contractile tension against which the abilities of NE and acetylcholine (ACh) to relax them was measured. Rings were contracted with 3.5 x 10^{-4}M AEP and once a stable contraction was obtained, NE was added in a cumulative fashion in

half-log increments. The rings were re-equilibrated and relaxations to ACh were measured in the same manner. Relaxations were reported as a percent of the initial AEP contraction. The relaxation of coronary artery rings to ACh is completely endothelium dependent, so the lack of relaxation to ACh was evidence of successful endothelium removal.

E. Data Collection and Statistical Methods for the Coronary Artery Studies

Data from all experiments with the coronary artery were expressed as mean $\pm$ SEM. In cases in which the same experiment was performed on more than one artery ring from the same rabbit, the mean value was used. EC_{50} values were calculated from curve fitting for the dose response curve of each experiment using the Boomer Program (version 2.5.2, Dr. David W.A. Bourne, 1992). The Student t test was used to test the statistical significance. $P \leq 0.05$ was considered significant.

F. Determination of the Effects of Ricin on Contractions of the Electrically Stimulated Papillary Muscle.

Ventricular papillary muscles were dissected free and mounted in 40 ml tissue baths containing oxygenated Tyrodes solution (140 mM NaCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 5.0 mM KCl, 5.0 mM HEPES, 5 mM NaOH, 10.0 mM glucose, and 0.5 mM aspartic acid at a pH of 7.35) equilibrated with 100% O_2 maintained at 37°C. Each papillary muscle was attached to a Grass isometric force-displacement transducer (FT03B), coupled to a Grass polygraph (Model 5) for recording, and stimulated via bipolar punctate platinum electrodes with 2-ms square-wave pulses of two times threshold voltage at a frequency of 1 Hz. Muscle length was varied initially to determine the length-tension curve. Length was then adjusted to provide twitch tension equal to 70% of the maximum. A period of at least 1 hour was allowed to elapse to ensure stable performance of the tissues. Force development was continuously recorded with stimulation frequency increments from 0.5 to 3 Hz on a Grass polygraph. Papillary muscle mass and length were determined and active tension development was expressed as grams/cross-sectional area (mm^2).

G. Determination of the Effects of Ricin on Contractions of the Electrically Stimulated Papillary Muscle in the Presence of Isoproterenol.

Papillary muscles were prepared as in "F", and the experiments were done as before, but this time in the presence of the ß-adrenoceptor agonist isoproterenol. Dose-response relationships for isoproterenol (given in 0.5 log unit increments from 10^{-9} to 10^{-7} M) were obtained from cumulative addition of the drug to the tissue baths. The response to each concentration was allowed to plateau over a 15-min interval before further addition of the drug to the bath. The increased active tension

development observed with the administration of isoproterenol was expressed as a percentage of the maximum tension generated by paired pacing.

H. Determination of the Effects of Ricin on Contractions of the Electrically Stimulated Papillary Muscle in the Presence of Bay K 8644.

Experiments were done as in "G" above, except in the presence of an opener of L-type calcium channels, 10^{-9} to 10^{-6} M Bay K 8644.

I. Determination of the Effects of Ricin Administration on the Electrophysical Properties of Rabbit Papillary Muscle.

Rabbit hearts from control and treated rabbits were placed into Krebs solution containing the following mM composition: NaCl, 118.5; $MgCl_2$, 1.2; KCl, 4.7; d-glucose, 11; $NaHCO_3$, 23.8; $CaCl_2$, 2.5; and KH_2PO_4, 1.2. Rabbit right ventricular papillary muscles with some attached ventricular wall were placed in a chamber with constant superfusion of Krebs solution, which was maintained at 38°C and aerated with 95% O_2-5% CO_2. The papillary muscles were electrically driven at 60 beats/min via platinum electrodes by rectangular impulses of 1 ms duration and twice threshold intensity delivered from a stimulator.

Excitability was determined by measuring the electrical threshold. The effective refractory period was measured by applying a test stimulus at three times threshold intensity at various times following the driving impulse. Every tenth pacing stimulus was followed by an extra stimulus placed within the absolute refractory periods, and moving out by 10 ms increments until the first extra beat appears.

Action potentials were recorded from the papillary muscle by conventional glass microelectrodes filled with 3 M KCl (impedance 10-15 megaohm) and coupled to a high impedance DC amplifier. The action potential was monitored continuously on a dual beam oscilloscope and recorded. The depolarization phase of the action potentials was differentiated with an analogue differentiator and the maximum rate of rise of the action potential V_{max} was also recorded.

J. Determination of the Effects of Ricin Administration on the Monoamine Oxidase Activity of the Papillary Muscle.

Determination of monoamine oxidase (monoamine:O_2 oxidoreductase [deaminating] EC 1.4.3.4.) activity in papillary muscles was by the method of Wurtman and Axelrod (1963). Fifty mg of each tissue was homogenized in 3 ml of 0.15 M KCl by a Polytron homogenizer at a setting of 2. The homogenate then was centrifuged at 1,000 g for 20 min in a refrigerated centrifuge (4°C). The supernatant was used for MAO assay. The reaction was started by incubating 100 µl of 0.5 M phosphate

buffer (pH 7.5), 50 µl of 0.05 µCi [^{14}C] tryptamine bisuccinate (specific activity 55.2 Ci/mmole). Two kinds of blanks were used: a boiled enzyme (90°C, 5 min) and a zero time blank to which 0.2 ml of 2 N HCl was added at zero time, i.e. before incubation. The reaction was stopped and the solution was made acidic with 0.2 ml of 2 N HCl. The metabolite, indoleacetic acid, was extracted with 4 ml of toluene during vortexing for 30 seconds. The organic phase was separated from the aqueous phase by centrifugation at low speed for 5 min. Two ml of the organic phase were collected in a 20 ml counting vial and mixed with 5 ml of scintillation cocktail, ScintiVerse™, for radioactivity determination. Data were expressed as nanomoles of indoleacetic acid/g wet weight of tissue/min.

K. Determination of the Effects of Ricin Administration on the Affinity and Density of ß-Adrenergic Receptors in the Rabbit Myocardium.

Rabbit myocardium was placed in an ice-cold buffer (5 mM Tris-HCl and 1 mM $MgCl_2$, pH 7.4), minced with scissors and homogenized 3 times with a Brinkman Polytron for 20 sec at a setting of 8. The homogenate was filtered through 4 layers of gauze. The filtrate was centrifuged at 500 g for 10 min at 4°C. The supernatant was then centrifuged at 40,000 g for 30 min at 4°C. The resulting pellet was resuspended in buffer (50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM EDTA, pH 7.5) and used in the binding assays.

The radioligand binding in the membrane preparations was determined by incubating 100 µl of the suspended pellet with various concentrations of [^{3}H]-dihydroalprenolol, a beta-adrenergic antagonist, in a final volume of 150 µl for 60 min at 38ºC in a shaking water bath (150 cycles/min). The reaction was terminated by adding 4 ml of ice-cold incubation buffer, and the reaction mixture was filtered rapidly through GF/C glass fiber filters (Whatman, Clinton NJ) on a manifold under vacuum. The filters were washed twice with 4 ml of ice-cold incubation buffer at room temperature and counted in a liquid scintillation counter. Non-specific binding was determined in the presence of 10 µM propranolol. All assays were done in duplicate. Protein content was determined by the Bio-Rad method.

L. Determining the Effects of Ricin Administration on Cardiac Performance in the Isolated Perfused Rabbit Heart

New Zealand White rabbits weighing between 2.0 and 3.0 kg were given a MLD dose of ricin, or sham injected. At forty-eight hours, tissues were taken. To prevent blood coagulation, 200 U/kg of sodium heparin was injected intravenously. The rabbits were anesthetized by ether inhalation, euthanized by a blunt blow to the head and decapitated. The thoracic cage was opened, and the heart was quickly excised and perfused retrogradely at 37°C using a constant flow pump (Masterflex, Cole-Parmer Instruments, Chicago, IL) through a plastic cannula inserted into the aorta.

The perfusate was a modified Tyrodes solution. Oxygen tension and pH were measured by a blood gas analyzer. After initiation of coronary perfusion, a small plastic tube was inserted into the left ventricle through an apical puncture to drain the Thebesian flow. The O_2 tension of the perfusate from the pulmonary artery was also measured by the blood gas analyzer. A latex balloon connected to a short polyethylene tube (10 cm long) was inserted into the left ventricle through the A-V valve. The left and right atria were tied around the tube to prevent the balloon from prolapsing. The balloon was then filled with bubble-free water, and the left ventricular pressure (LVP) generated was measured with a Statham P23 ID transducer (Statham Instruments, Oxnard, CA) attached to the polyethylene tube. Coronary perfusion pressure (CPP) was measured throughout the experiment. The electrocardiogram (ECG) was obtained using epicardial hook-shaped electrodes attached to the right ventricular outflow and the LV apex proximately. The perfusion flow rate was adjusted to about 100 mm Hg at baseline, and the perfusion flow was fixed there, thereafter. The balloon volume was adjusted as the LV end-diastolic pressure (LVEDP) was set to 5 to 10 mm Hg.

After a stable condition was attained (in about 30 min) with oxygenated modified Tyrodes perfusion, baseline measurements of CPP, coronary flow, and ECG were made. The balloon volume was then adjusted from 0.3 ml to 1.7 ml with 0.2 ml increments, and the LVP, LVEDP, +dp/dt and - dp/dt were recorded at each step, in triplicate for each rabbit heart. Permanent records were obtained on a Grass model 7 polygraph.

In seventeen rabbit hearts (9 control and eight from rabbits given ricin 48 hrs earlier), isoproterenol was incrementally added to the perfusate as follows: 10^{-9}, 3.2×10^{-9}, and 10^{-8} M. Hemodynamic measurements were repeated at each isoproterenol concentration. All parameters were recorded after a stable condition was attained in 10 min at each dose-step in each group.

The experiment was continued after a 30 minute washout period. The perfusate was switched from the control modified Tyrodes to one containing 10^{-7} M propranolol to block any possible beta-adrenoceptor stimulation by phenylephrine. Stepwise phenylephrine perfusion was then started as follows: 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M with a 10 minute stabilization interval between concentrations. The concentration of propranolol in the perfusate was maintained at 10^{-7} M throughout the phenylephrine infusion. At the end of the experiment, the heart was detached from the perfusion apparatus. The left and right ventricles were lightly blotted and weighed.

M. Statistical Methods for the Isolated Heart and Papillary Muscle.

Data for the isolated heart and papillary muscle were compared using the Student t Test. Values of $P \leq 0.05$ were considered significant.

III. RESULTS

A. Effects of Ricin Administration on Coronary Artery Contractions

All of the coronary artery rings contracted to serotonin, histamine, AEP and potassium. Concentration-response curves were constructed for serotonin with (Fig 1) and without (Fig 2) endothelium, and histamine with (Fig 3) and without (Fig 4) endothelium. In each case ricin administration altered responses of the artery rings to the agonists. It also altered the contractions obtained to the single concentrations of AEP and potassium.

Ricin administration significantly increased ($P < 0.05$) the EC_{50} of the coronary artery to 5-HT and histamine, both with the endothelium intact and with it removed (Table 1).

Ricin administration significantly ($P < 0.05$) increased the maximal tension achieved by the coronary artery with either endothelium present or removed to 3 of the 4 agonists tested, serotonin, histamine and 35 mM AEP (Table 2). The contractions of coronary artery to 55 mM potassium were also increased but only those with endothelium present to a significant extent.

B. Effects of Ricin Administration on Coronary Artery Relaxations

Contractions of the artery rings to AEP were well-maintained (not shown) and rings with endothelium present (Fig 5) or absent (Fig 6) relaxed well to NE. Rings with endothelium relaxed well to ACh (Fig 7), while those without endothelium did not relax to ACh (data not shown).

Ricin administration significantly decreased the EC_{50} of coronary artery with endothelium present to NE (Table 3), but did not significantly alter the EC_{50} of coronary artery with the endothelium removed. Ricin administration had no effect on the EC_{50} of the coronary artery to ACh.

Ricin administration increased the maximal relaxations of rabbit coronary arteries to both ACh and NE but neither to a statistically significant extent (Table 4).

C. Effects of Ricin on Contractions of Paced Papillary Muscle.

Responses of papillary muscle from rabbits given ricin were compared with those of sham injected control rabbits. Time to peak tension and duration of papillary muscle contractions did not differ between the two groups (Fig 8). Frequency responses of papillary muscle paced at from 0.5 to 3.0 Hz were higher in papillary muscle from ricin-treated rabbits, but not significantly ($P > 0.05$) (Fig 9). Figure 10 shows the effects of ricin on contractions of rabbit papillary muscle stimulated by 1 Hz and by paired pacing. There was no difference in either case between rabbits receiving ricin and control rabbits.

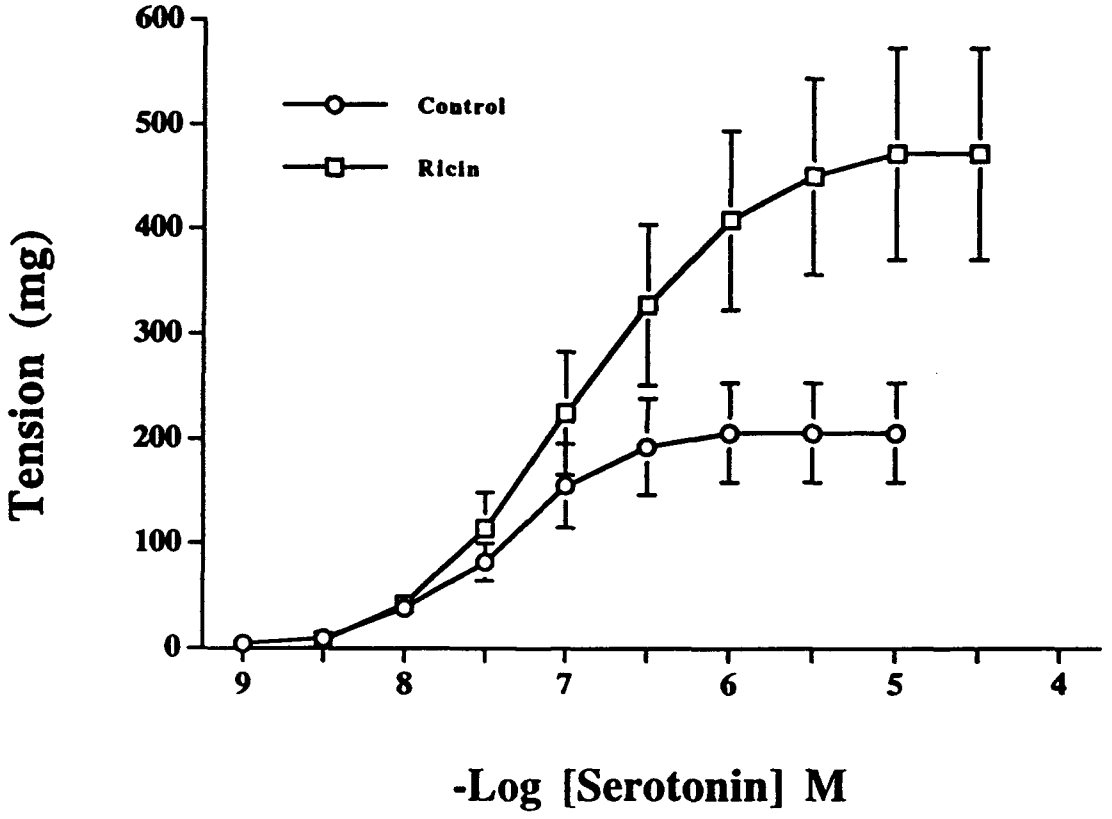

Fig 1. Contractions of rabbit coronary artery rings with endothelium intact 48 hr after i.v. injection of 0.22 μg/kg of ricin. Each point is the mean ± SEM of rings from 7-8 rabbits.

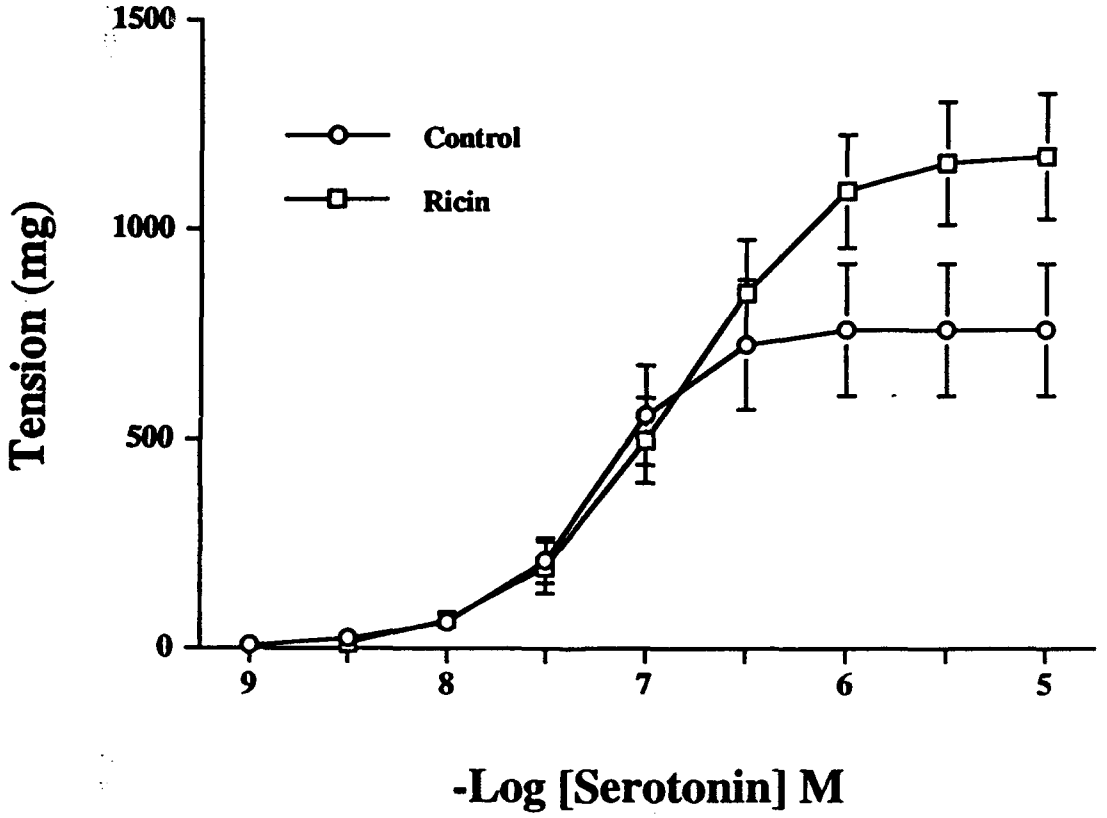

Fig 2. Contractions of rabbit coronary artery rings with endothelium removed 48 hr after i.v. injection of 0.22 μg/kg of ricin. Each point is the mean ± SEM of rings from 7 rabbits.

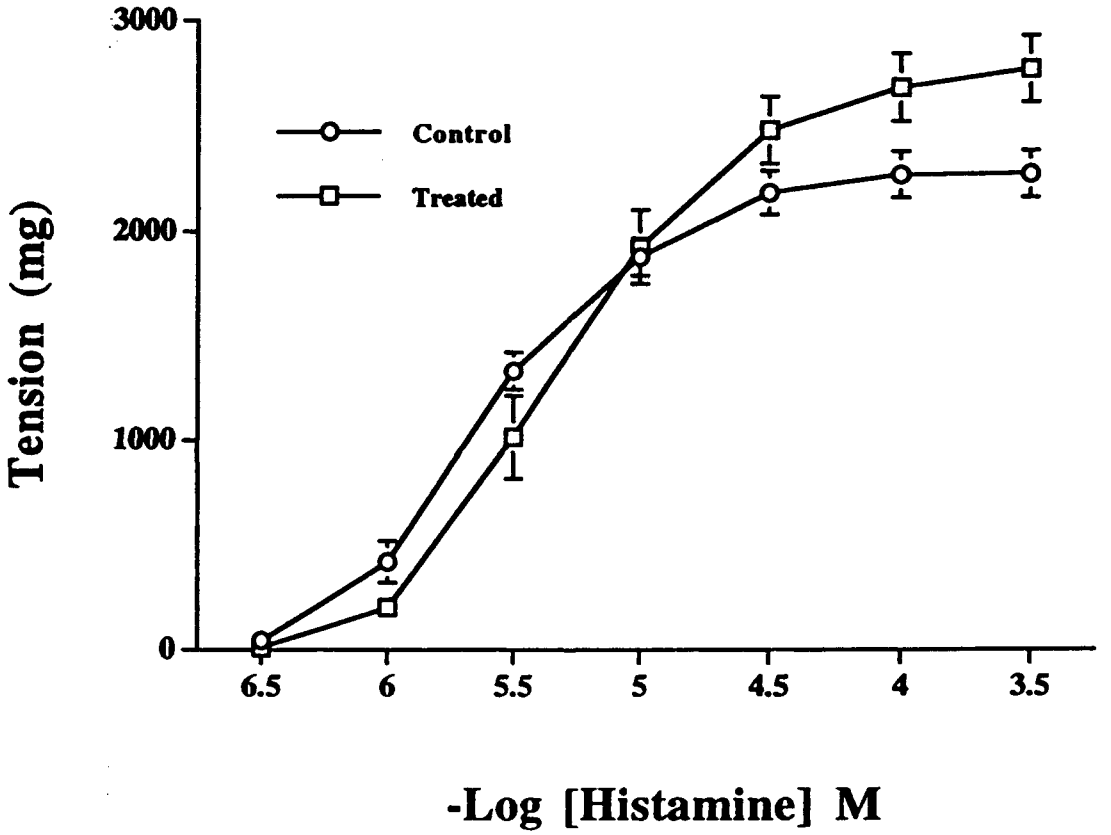

Fig 3. Contractions of rabbit coronary artery rings with endothelium intact 48 hr after i.v. injection of 0.22 μg/kg of ricin. Each point is the mean ± SEM of rings from 8 rabbits.

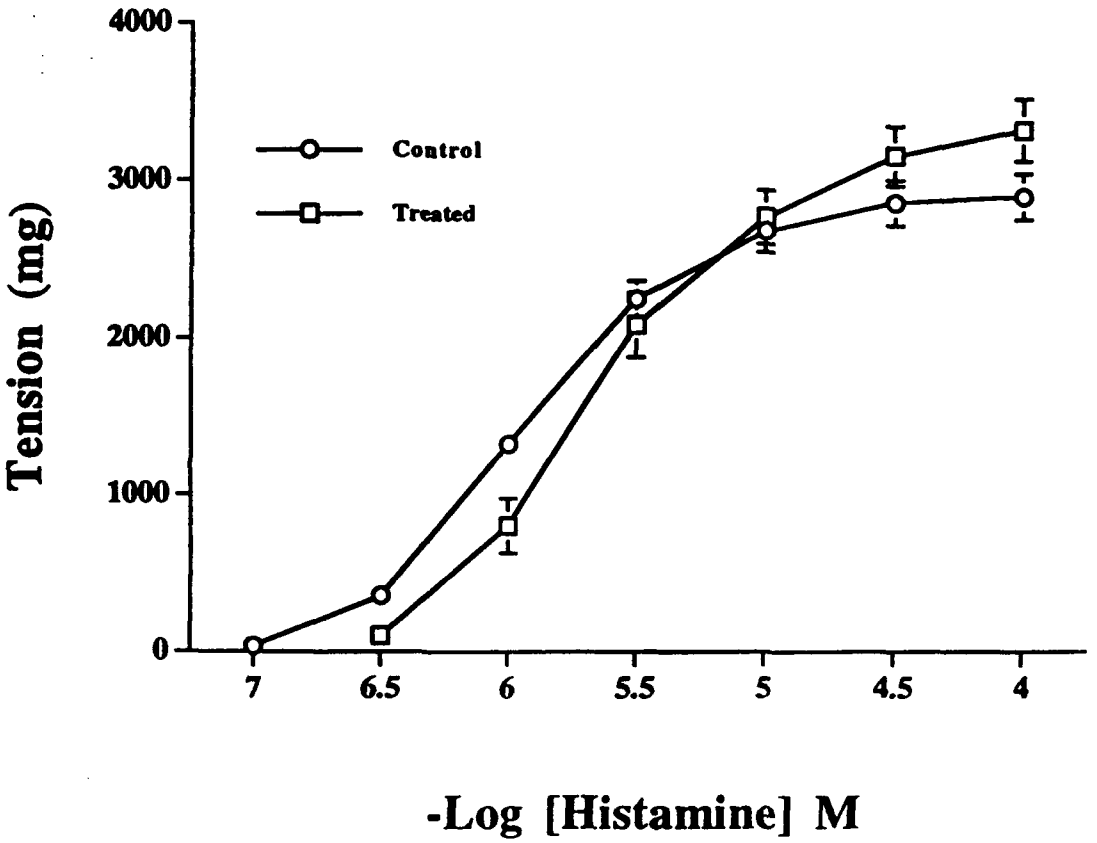

Fig 4. Contractions of rabbit coronary artery rings with endothelium removed 48 hr after i.v. injection of 0.22 μg/kg of ricin. Each point is the mean ± SEM of rings from 8 rabbits.

Table 1. The EC_{50} for contraction by agonists of coronary artery rings from rabbits receiving ricin and from control rabbits.

	EC_{50} ($x10^{-8}M$)			
	Control		Ricin (0.22 μg/kg)	
	endothelium present	endothelium removed	endothelium present	endothelium removed
Agent	Mean±SEM (n)	Mean±SEM (n)	Mean±SEM (n)	Mean±SEM (n)
5-HT	4.70±0.49 (7)	7.01±1.05 (8)	10.8±1.64* (7)	15.4±2.50* (8)
Histamine	240±27.4 (8)	106±9.42 (8)	460±67.7* (8)	230±14.9** (8)

n= the number of rabbits. * Significantly different from corresponding control at $P < 0.05$; ** at $P < 0.01$.

Table 2. The maximal tension to adonists of coronary artery rings from control rabbits and rabbits receiving ricin.

	Tension (g)			
	Control		Ricin (0.22 μg/kg)	
	endothelium present	endothelium removed	endothelium present	endothelium removed
Agent	Mean±SEM(n)	Mean±SEM(n)	Mean±SEM(n)	Mean±SEM(n)
5-HT	0.19±0.04 (8)	0.73±0.14(8)	0.50±0.09*(8)	1.05±0.19*(8)
Histamine	2.22±0.11(9)	2.85±0.14(8)	2.80±0.14**(9)	3.52±0.16**(8)
55 mM KCl	1.29±0.12(7)	1.69±0.11(7)	1.73±0.14*(9)	2.05±0.16(9)
35 mM AEP	1.76±0.12(8)	2.20±0.12(9)	2.18±0.11*(9)	2.66±0.12*(9)

n= the number of rabbits. * Significantly different from corresponding control at $P < 0.05$; ** $P < 0.01$.

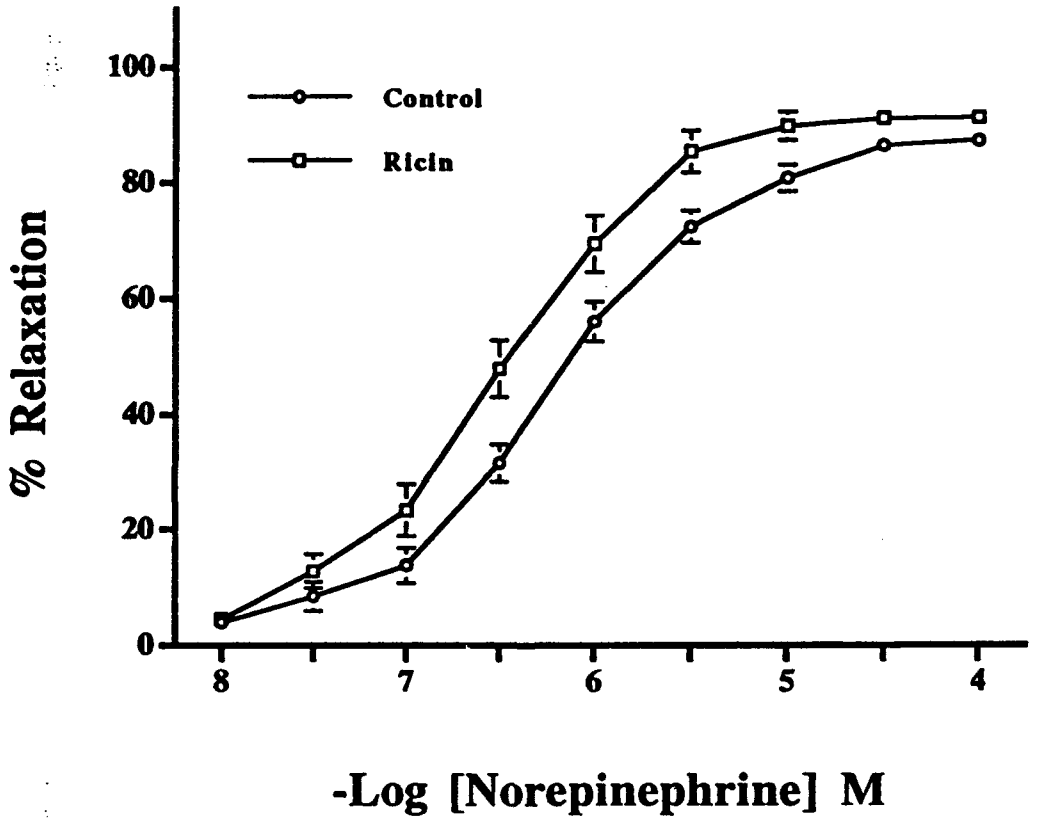

Fig 5. Relaxations to NE of rabbit coronary artery rings with endothelium intact, contracted with AEP, 48 hrs after i.v. injection of 0.22 μg/kg of ricin. Each point is the mean ± SEM of rings from 7-8 rabbits.

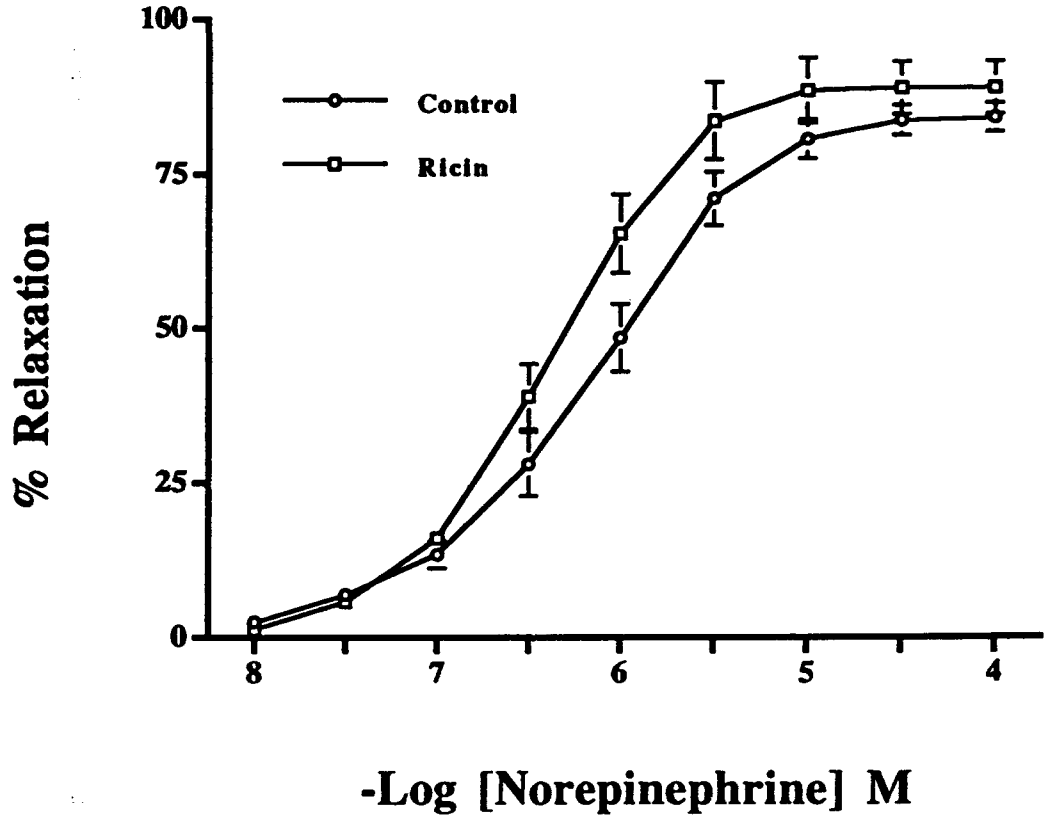

Fig 6. Relaxations to NE of rabbit coronary artery rings with endothelium removed, contracted with AEP, 48 hrs after i.v. injection of 0.22 μg/kg of ricin. Each point is the mean ± SEM of rings from 7 rabbits.

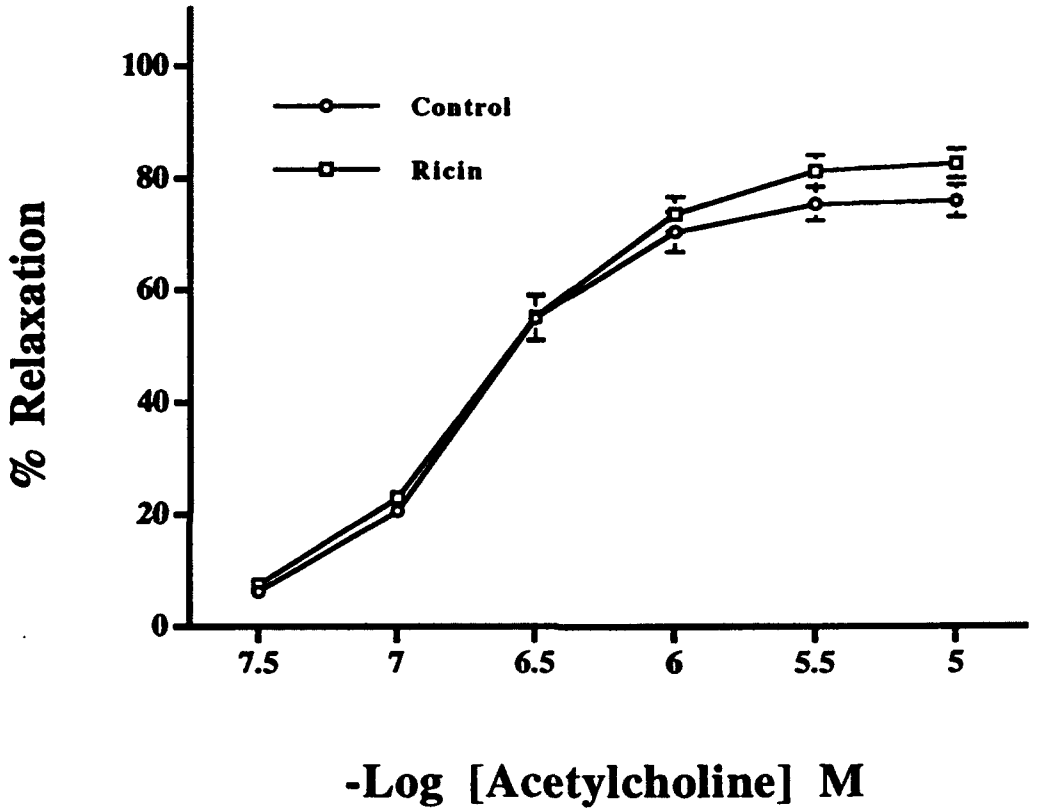

Fig 7. Relaxations to ACh of rabbit coronary artery rings contracted with AEP, 48 hrs after i.v. injection of 0.22 μg/kg of ricin. Each point is the mean ± SEM from 7-8 rabbits.

Table 3. The EC_{50} for relaxation by agonists of AEP-contracted coronary artery rings from rabbits receiving ricin and control rabbits.

	EC_{50} ($x10^{-8}M$)			
	Control		Ricin (0.22μg/kg)	
	endothelium present	endothelium removed	endothelium present	endothelium removed
Agent	Mean±SEM (n)	Mean±SEM (n)	Mean±SEM (n)	Mean±SEM (n)
NE	77.2±9.51(7)	65.4±10.8(7)	24.2±4.79*(7)	40.4±3.44(7)
ACh	21.3±0.97(6)	_____	21.0±2.90(8)	_____

n= the number of rabbits. * Significantly different from corresponding control at P < 0.01.

Table 4. The maximal relaxation to NE or ACh of the rabbit coronary artery precontracted with 3.5×10^{-4} M AEP.

	% of Initial Contraction			
	Control		Ricin (0.22 μg/kg)	
	endothelium present	endothelium removed	endothelium present	endothelium removed
Agent	Mean±SEM (n)	Mean±SEM (n)	Mean±SEM (n)	Mean±SEM (n)
NE	87.4±1.2 (7)	84.2±2.3 (7)	89.3±2.5 (8)	89.0±4.2 (7)
ACh	76.0±2.9 (7)	———	82.7±2.6 (8)	———

n= the number of rabbits. No values were different at $P < 0.05$.

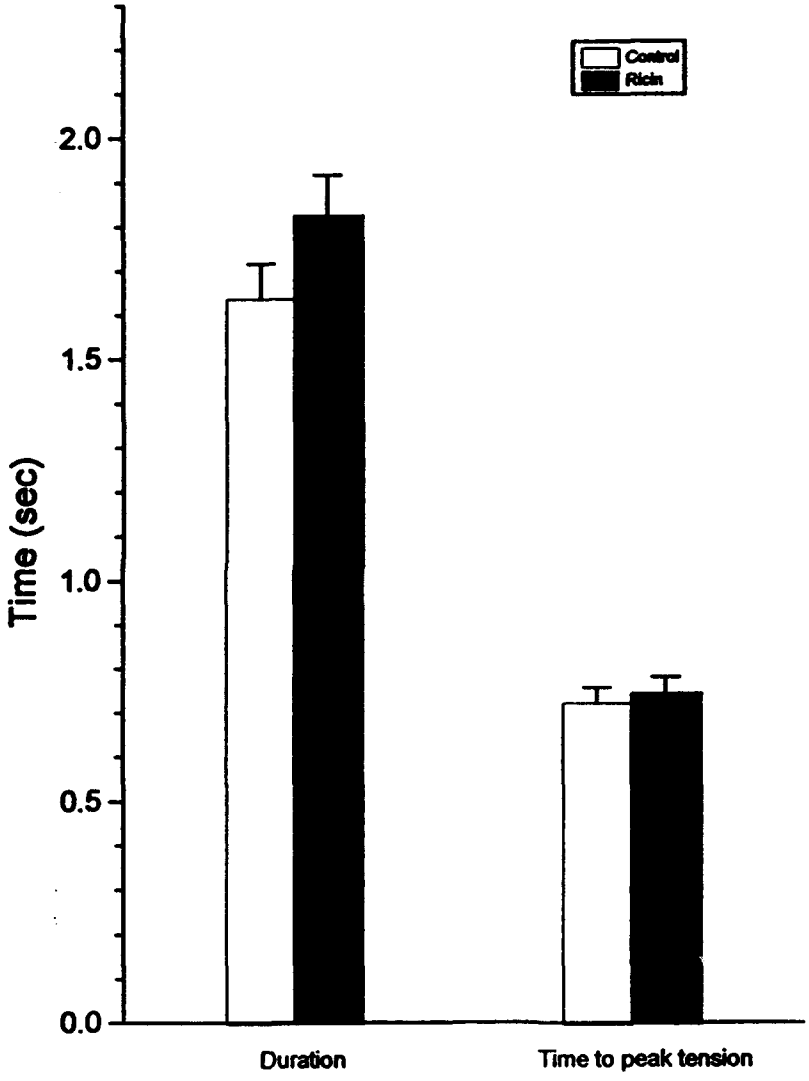

Fig 8. Effects of ricin on the rabbit papillary muscle contraction duration, and time to peak tension. Each bar is the mean ± SEM from six rabbits.

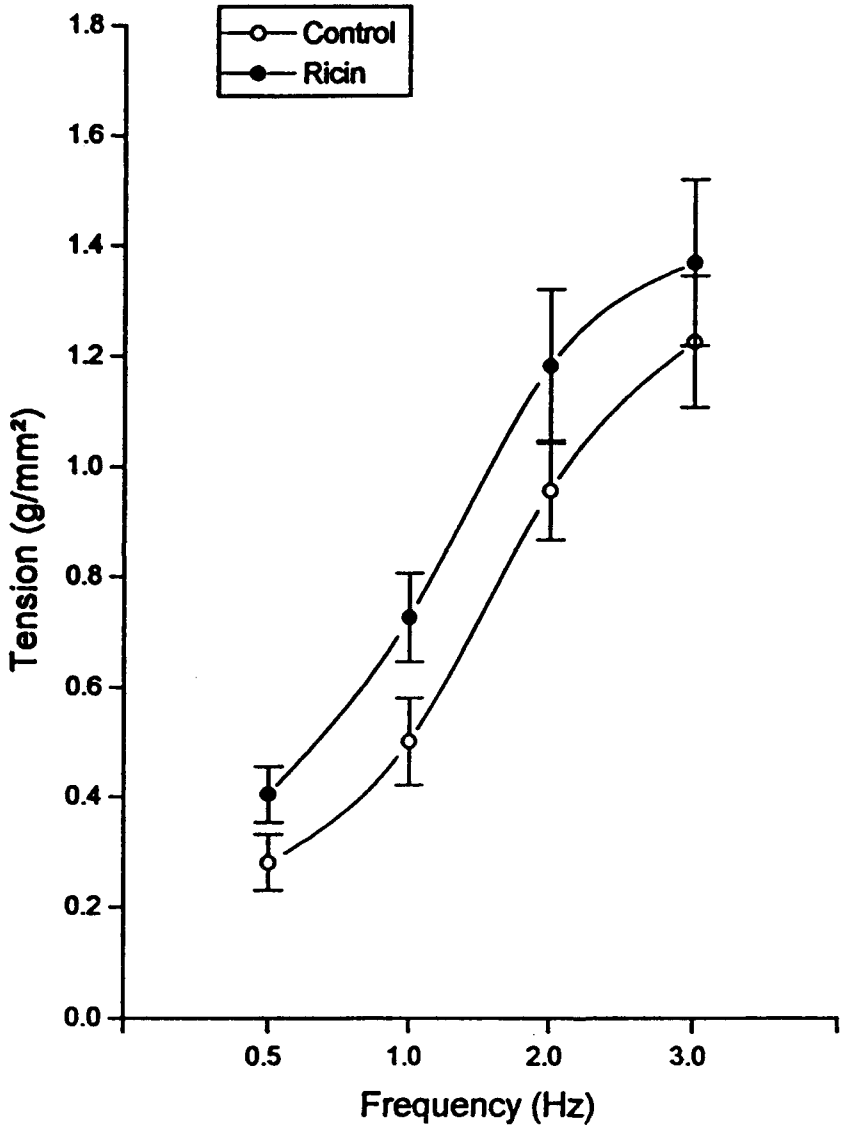

Fig 9. Effects of ricin on contractions of rabbit papillary muscle to electrical stimulation. Each point represents the mean ± SEM from 6 rabbits.

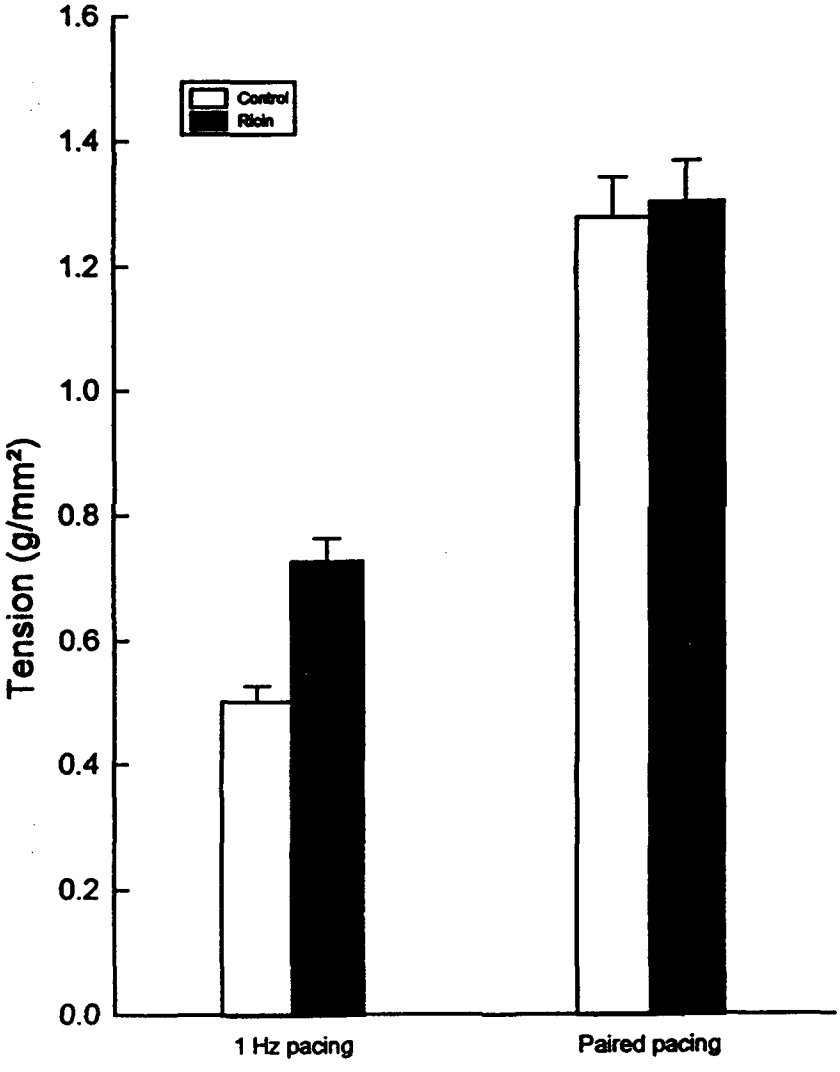

Fig 10. Effects of ricin on contractions of rabbit papillary muscle stimulated by 1 Hz and by paired pacing. Each bar is the mean ± SEM from six rabbits.

D. Effects of Ricin Administration on Contractions of Paced Papillary Muscle in the Presence of Isoproterenol.

Papillary muscle paced at 1 Hz was exposed to cumulatively increasing concentrations of isoproterenol (Fig 11). No differences in contractions were significant ($P > 0.05$).

E. Effects of Ricin Administration on Contractions of Paced Papillary Muscle in the Presence of Bay K 8644.

Papillary muscle paced at 1 Hz was exposed to cumulatively-increasing concentrations of the calcium channel agonist, Bay K 8644 (Fig 12). In the control group, Bay K 8644 induced an increase in force of contraction that was half-maximal at $5.7 \times 10^{-8} \pm 2.9 \times 10^{-9}$ M compared to that induced in papillary muscle from rabbits given ricin ($3.3 \times 10^{-8} \pm 4.8 \times 10^{-9}$ M). The increased electrically stimulated contraction amplitude in the presence of Bay K 8644 was significant at $p=0.05$.

The maximal contraction to electrical stimulation obtained in the presence of Bay K 8644 was not altered by ricin.

F. Effects of Ricin Administration on the Electrophysiological Properties of Rabbit Papillary Muscle.

Electrophysiological parameters of rabbit papillary muscle were determined (Table 4). These included measuring the resting membrane potential, the action potential amplitude, the overshoot potential, the maximal dv/dt of Phase 0, the action potential duration, and the effective refractory period. In comparing papillary muscle data from rabbits receiving ricin and control rabbits, none of the values were different ($p > 0.05$).

G. Effects of Ricin Administration on Monoamine Oxidase Activity of Rabbit Papillary Muscle.

Papillary muscle from rabbits receiving ricin and sham injected rabbits both had detectable concentrations of MAO (Fig 13). Ricin increased the MAO activity of papillary muscle, mean ± SEM, compared to that of control rabbits (1.44 ± 0.09 nmol/g/min, n=6) (1.16 ± 0.04 nmol/g/min, $p < 0.05$, n=6).

H. Determination of the Effects of Ricin Administration on Binding of a Beta Receptor Agonist.

The [^{3}H]-dihydroalprenol binding in preparations from rabbits given ricin and control rabbits was compared. Ricin increased, but not significantly, the number of [^{3}H]-dihydroalprenolol binding sites, mean ± SEM ($B_{max} = 386.6 \pm 123.0$ fmol/mg, n = 6) in myocardium compared to

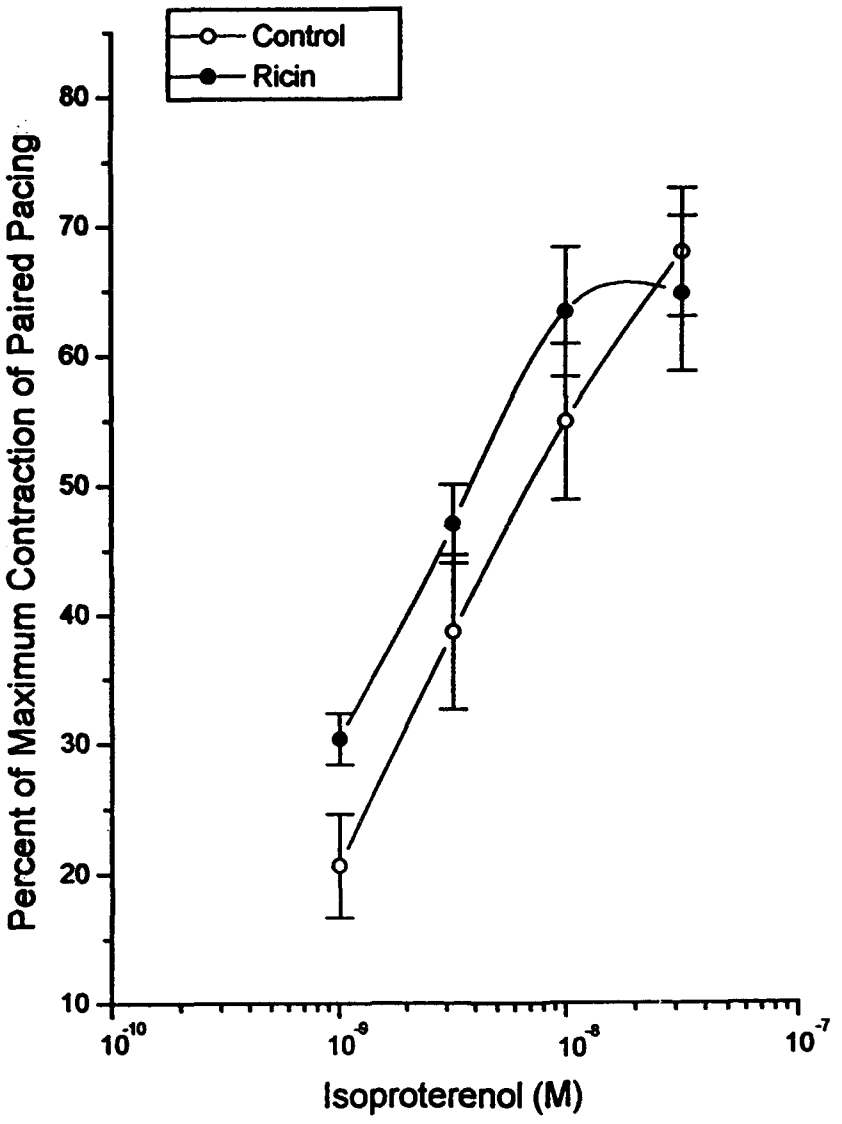

Fig 11. Effects of ricin on contractions of rabbit papillary muscle to 1 Hz electrical stimulation in the presence of isoproterenol. Each point represents the mean ± SEM from 6 rabbits.

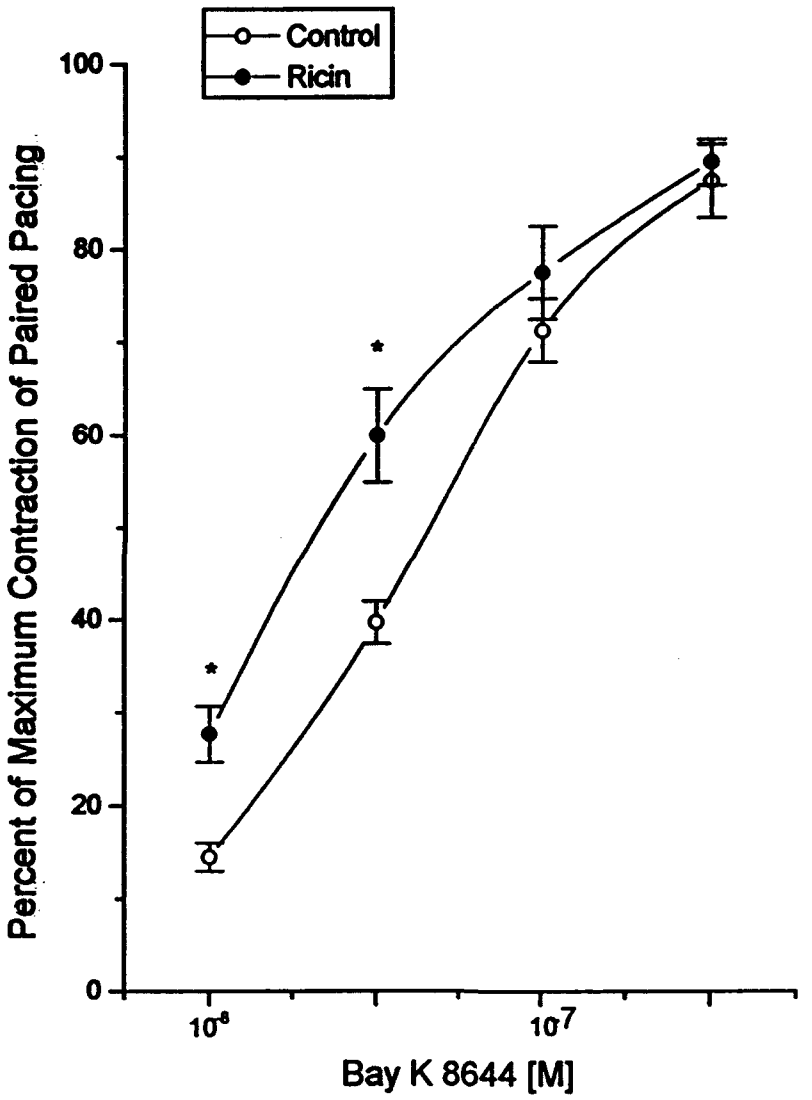

Fig 12. Effects of ricin on contractions of rabbit papillary muscle to 1 Hz electrical stimulation in the presence of Bay K 8644. Each point is the mean ± SEM from 6 rabbits. *Different from control at $p<0.05$.

Table 5: Electrophysiological parameters of rabbit papillary muscle from control rabbits and those given ricin 48 hours earlier.

	Control Mean ± SEM	Ricin Mean ± SEM
Resting Membrane Potential (mV)	-67 ± 1	-71 ± 2
Action Potential Amplitude (mV)	87 ± 4	93 ± 4
Overshoot Potential (mV)	20 ± 2	22 ± 3
Maximal *dv/dt* of Phase 0 (V/sec)	68 ± 4	71 ± 4
Action Potential Duration (ms)	128 ± 8	150 ± 7
Effective Refractory Period (ms)	125 ± 12	162 ± 12

n = 6 different rabbits.

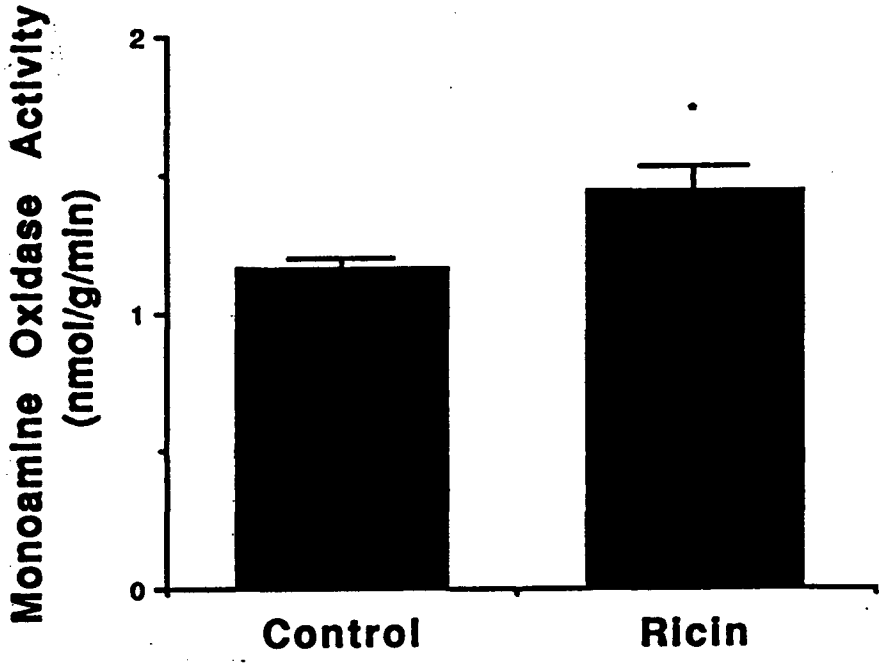

Fig 13. Effects of the administration of a minimal lethal dose of ricin 48 hours earlier on the monoamine oxidase activity of rabbit papillary muscle. Each point is the mean ± SEM of tissues from 6 rabbits. * = Different from control, $p < 0.05$.

control animals (B_{max}=328.3 ± 144.9 fmol/mg, n=6). An increase in number of binding sites is indicative of an increased number of beta adrenergic receptors.

Ricin did not alter the K_d for [^{3}H]-dihydroalprenolol binding, mean ± SEM, (Control: 9.1 ± 5.7 nM, n = 6; Ricin administered: 9.1 ± 2.0 nM, n = 6), indicating that ricin did not alter the affinity of the beta adrenergic receptor binding sites for dihydroalprenolol.

I. **Effects of Ricin Administration on Cardiac Performance in the Isolated Perfused Rabbit Heart.**

Administration of an intravenous bolus of a MLD of ricin 48 hours earlier did not alter (control, n = 10 vs. ricin, n = 8, mean ± SEM oxygen consumption (0.057 ± 0.006 vs. 0.054 ± 0.011 ml/min/gm), heart rate (150.16 ± 19.95 vs. 168.86 ± 25.75 ms), P-R interval (73.23 ± vs. 74.18 ± 8.66 ms), QRS duration (47.25 ± 4.73 vs. 45.58 ± 6.01 ms), or other electrocardiographic parameters. No arrhythmias or conduction abnormalities were observed during the baseline recording period. However, ricin administration significantly reduced left ventricular compliance (Fig 14), diminished the left ventricular developed pressure per LVED (Fig 15), and diminished the maximal left ventricular developed pressure (Fig 16) and maximal +dp/dt (Fig 17). The negative dp/dt (Fig 18) was not different in the control or ricin administered groups.

To assess the effects of ricin on beta adrenoceptor-mediated inotropic effects in the isolated rabbit heart, isoproterenol was incrementally added to the perfusate to generate dose response curves. Left ventricular developed pressure, +dp/dt and -dp/dt were recorded and there was no difference in those measurements between the control group and the one receiving ricin (Fig 19 and 20). (For -dp/dt, data are not shown.) The effects of ricin on alpha adrenoceptor-mediated inotropic effects were also evaluated. Propranolol was used to block possible beta adrenoceptor stimulation by phenylephrine. Concentration-dependent changes in the above hemodynamic parameters by phenylephrine were not affected by ricin administration (Fig 21 and 22). Thus, MLD ricin administration only reduced the diastolic and systolic function of the myocardium.

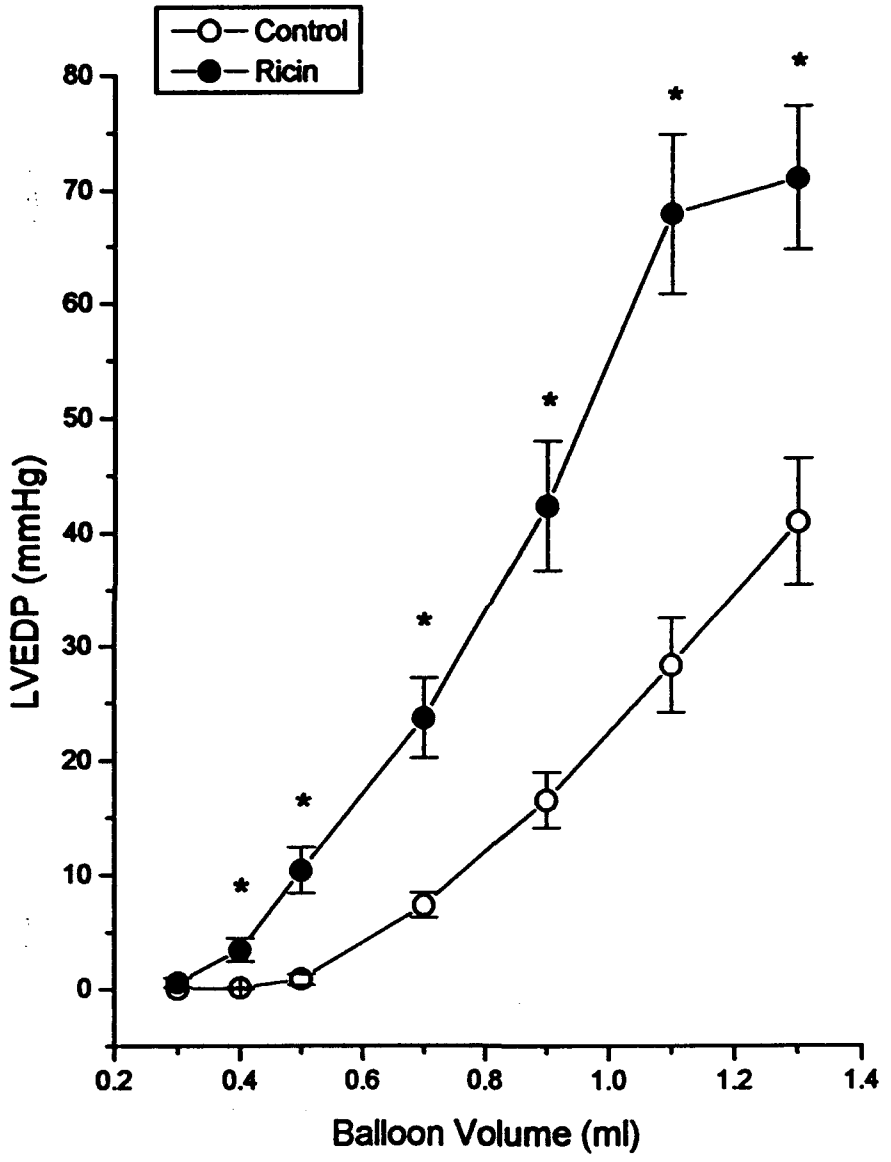

Fig 14. Effects of ricin on the left ventricular compliance of the rabbit heart. Each point is the mean ± SEM from 9 to 10 rabbits.
* different from control at $p<0.01$.

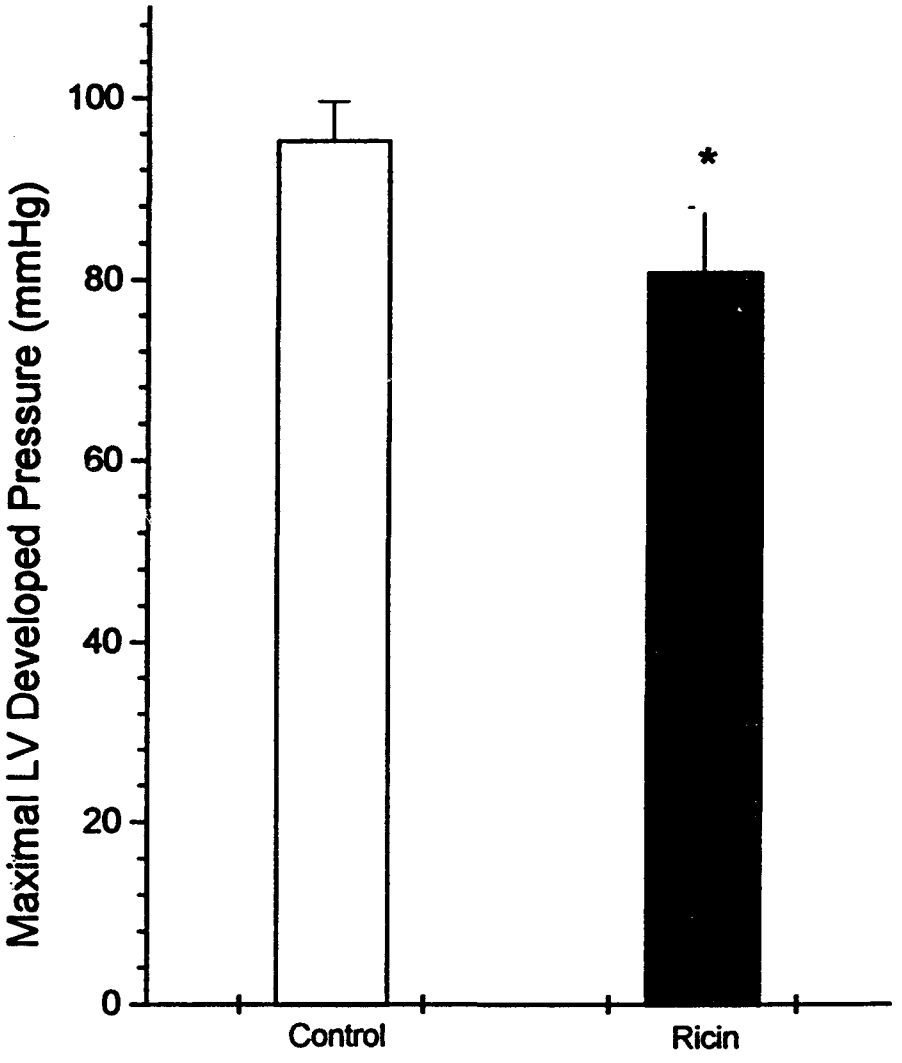

Fig 15. Effects of ricin on maximal left ventricular developed pressure of the rabbit heart. Each bar is the mean ± SEM from 9 to 10 rabbits. * Different from control at $p<0.05$.

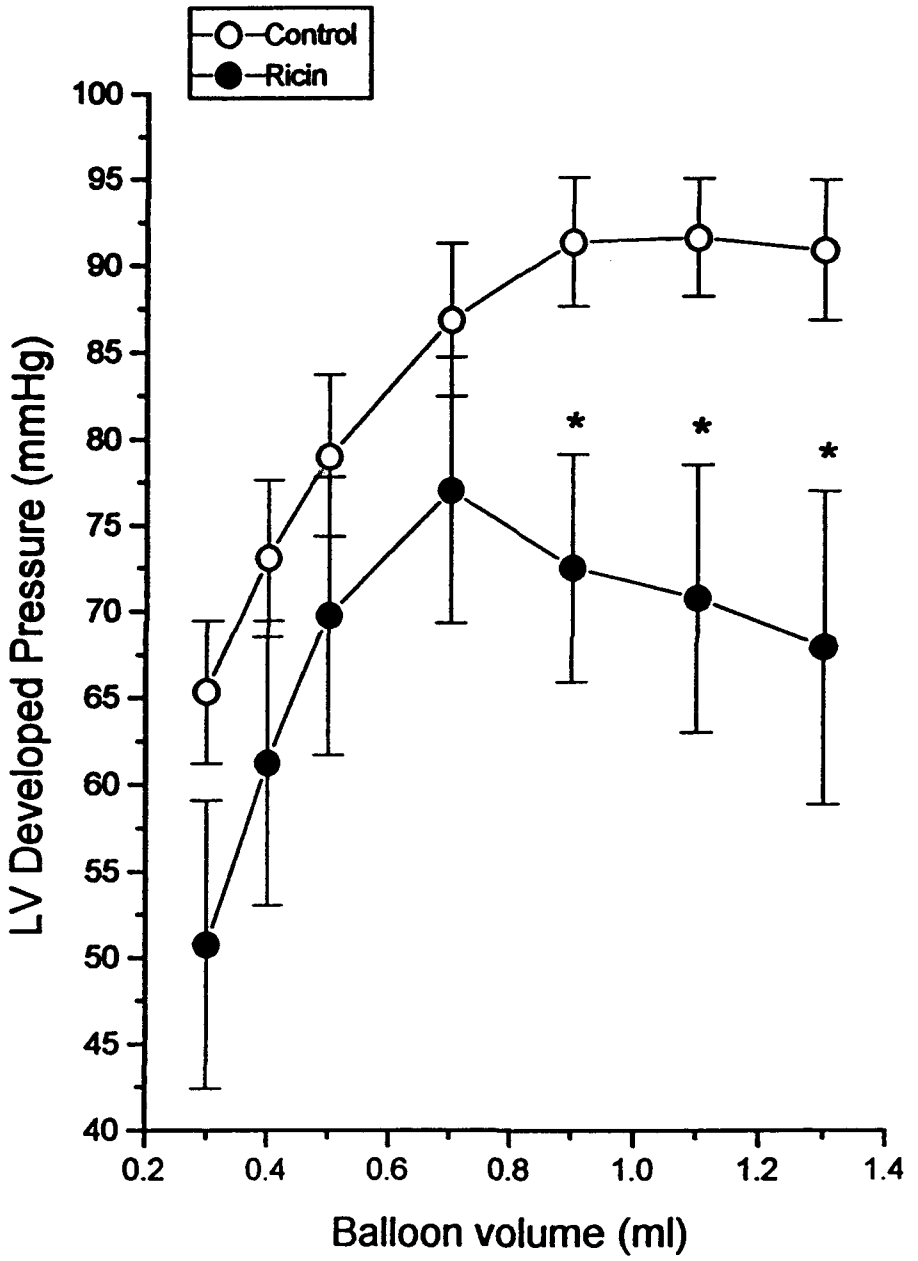

Fig 16. Effects of ricin on the left ventricular developed pressure per balloon volume. Each point represents the mean ± SEM from 9 to 10 rabbits. * Different from control at $p<0.05$.

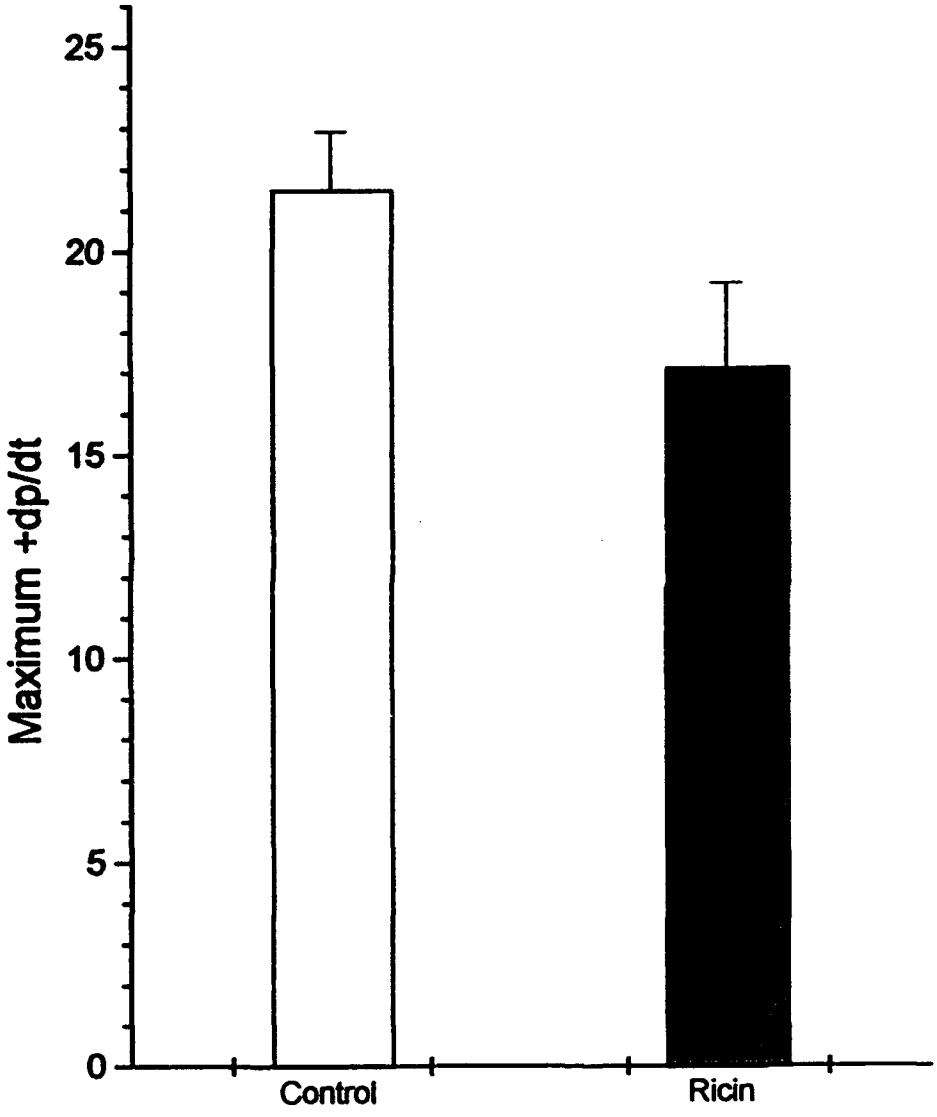

Fig 17. Effects of ricin on the contractility of the rabbit heart as reflected by the maximal +dp/dt at LVEDP. Each bar is the mean ± SEM from 9 to 10 rabbits.

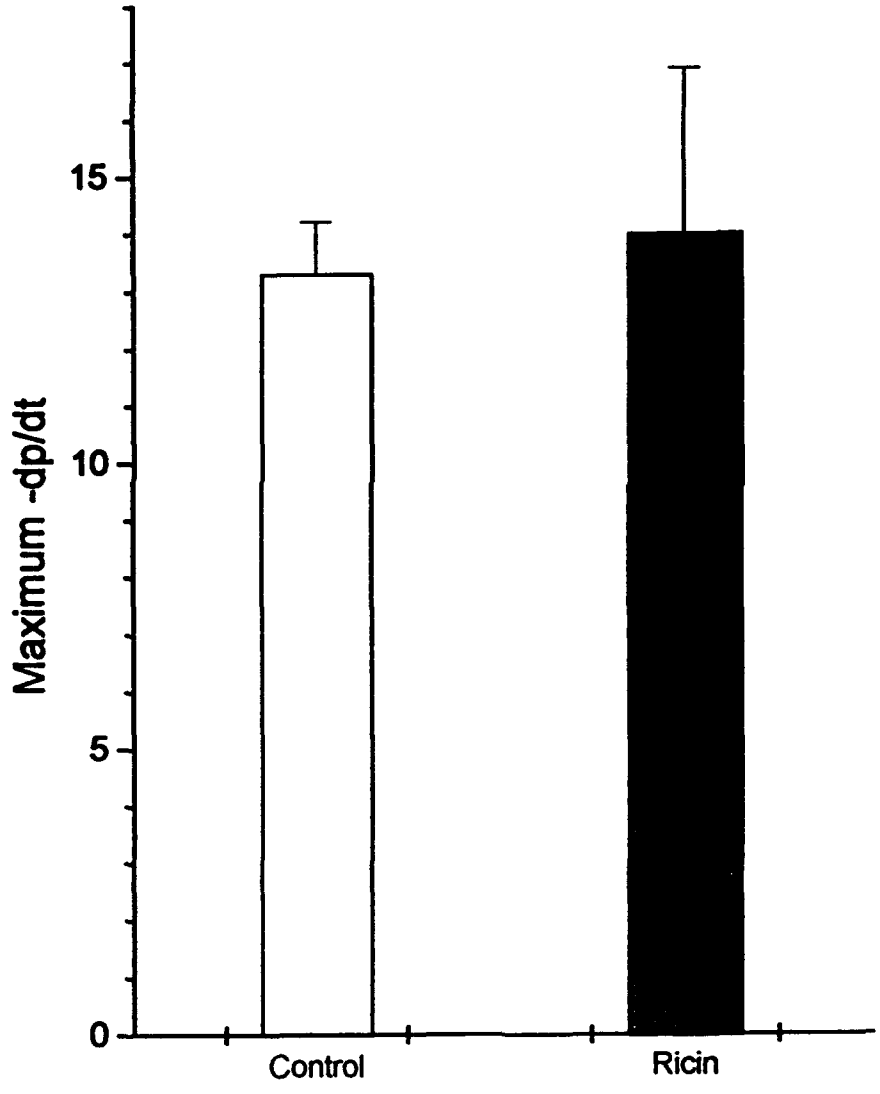

Fig 18. Effects fo ricin on the relaxation rate of the rabbit heart as reflected by the maximal -dp/dt. Each bar is the mean ± SEM from 9 to 10 rabbits.

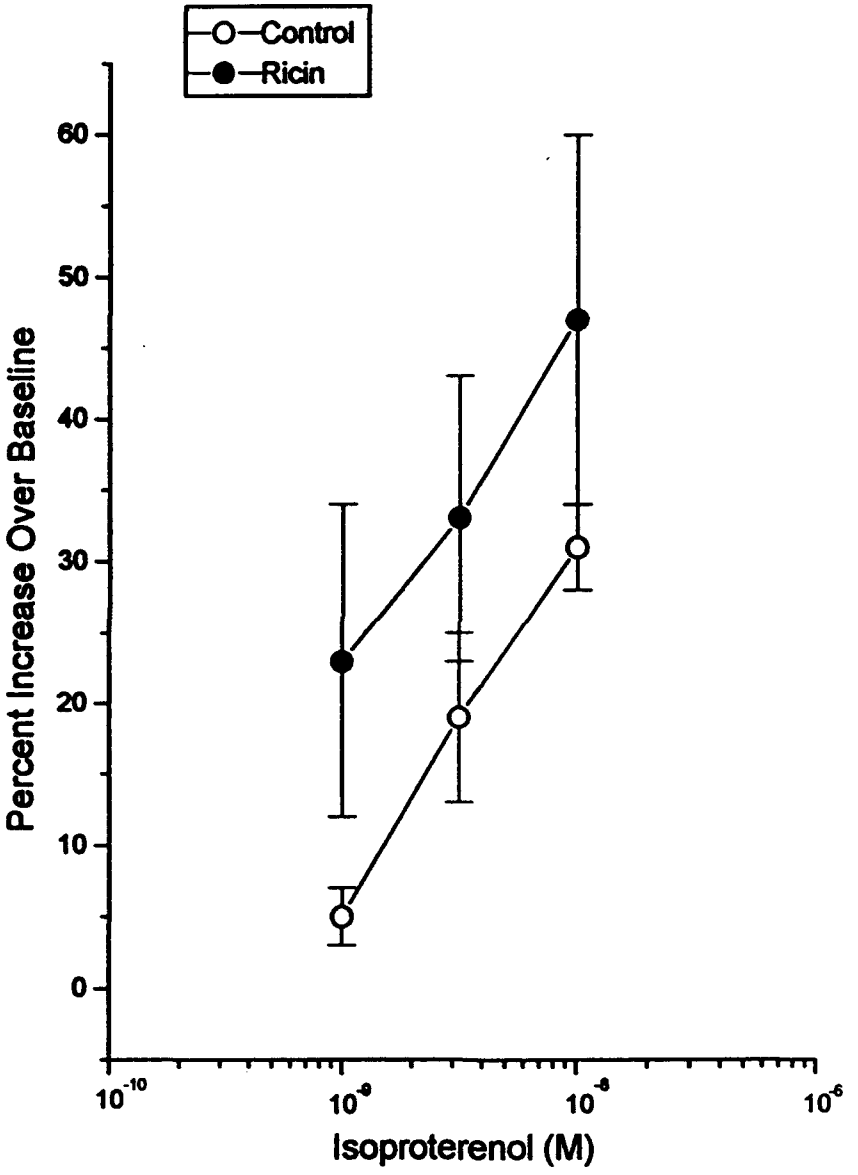

Fig 19. Effects of ricin on the isoproterenol-induced increase in left ventricular developed pressure. Each point is the mean ± SEM from 9 to 10 rabbits.

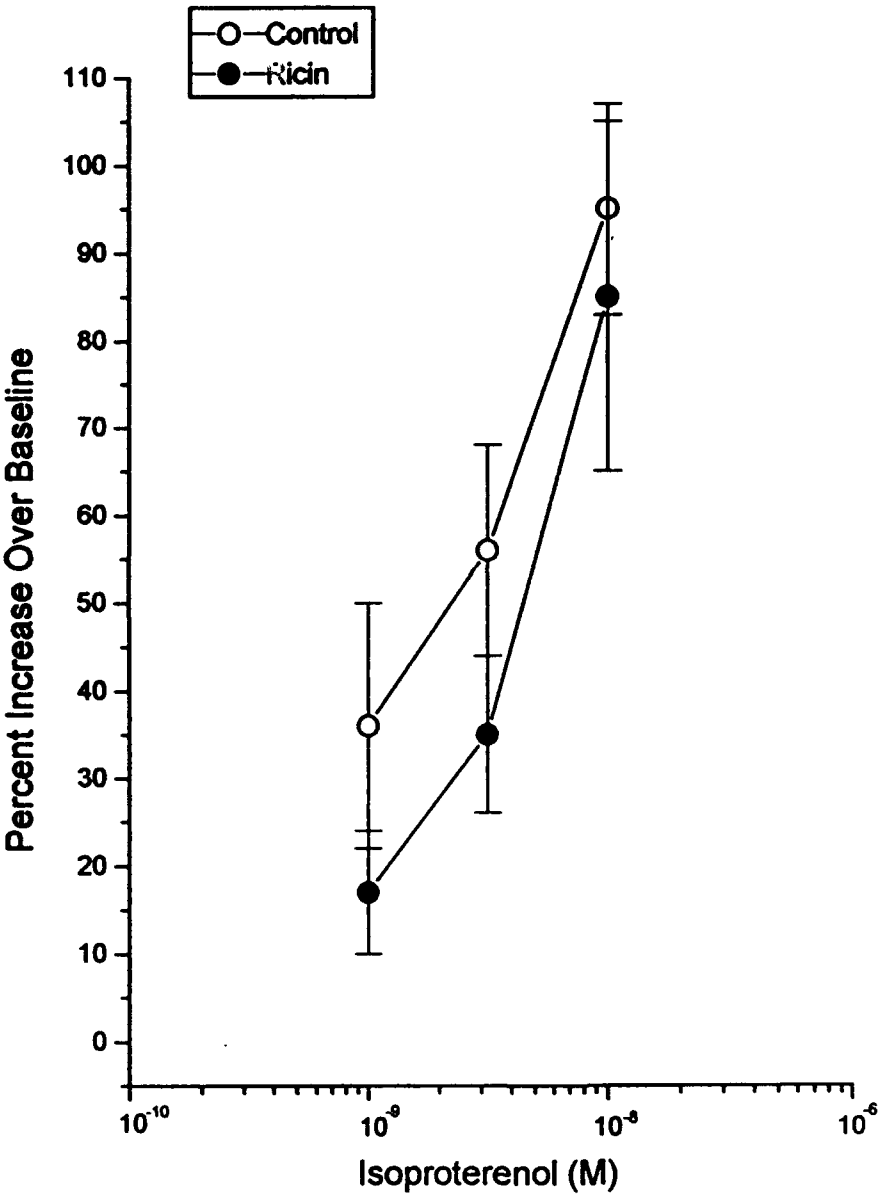

Fig 20. Effects of ricin on the contractility of the rabbit heart as reflected by +dp/dt as influenced by isoproterenol. Each point is the mean ± SEM from 9 to 10 rabbits.

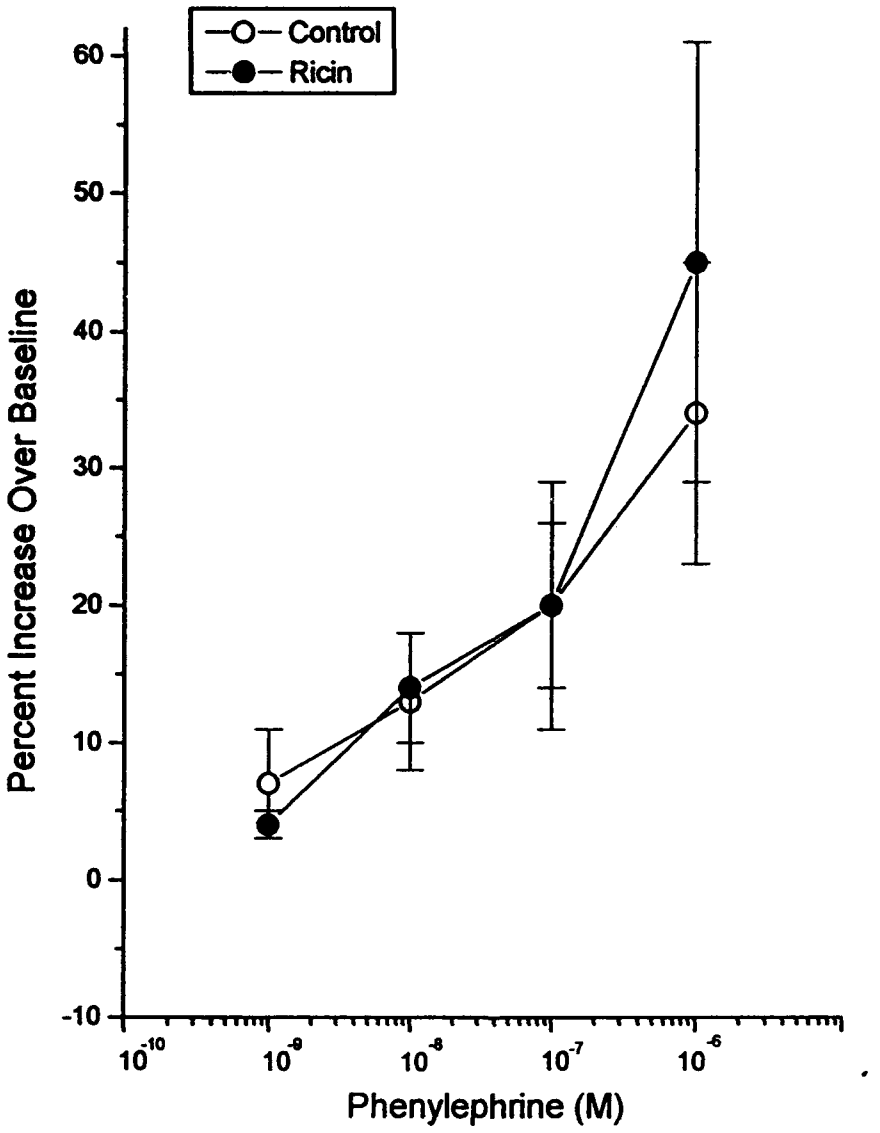

Fig 21. Effects of ricin on the phenylephrine-induced increase in left ventricular developed pressure. Each point is the mean ± SEM from 9 to 10 rabbits.

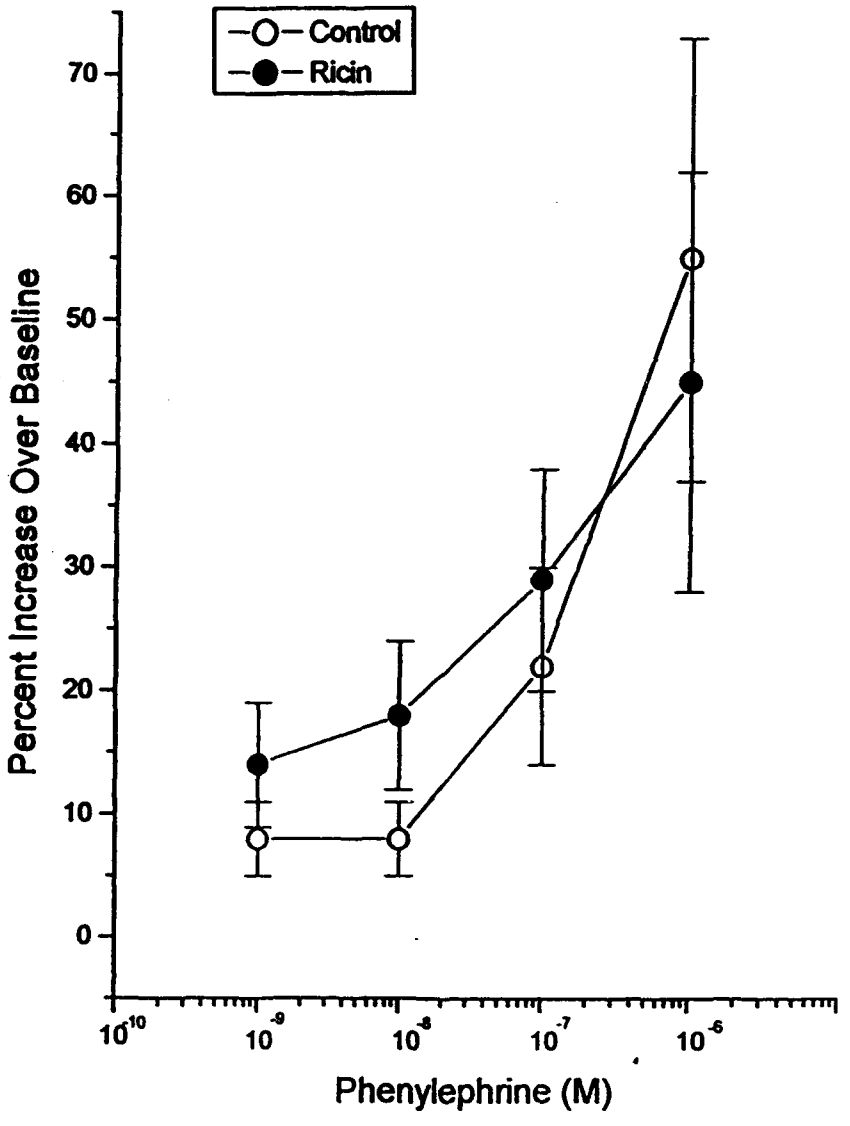

Fig 22. Effects of ricin on the contractility of the rabbit heart as reflected by +dp/dt as influenced by phenylephrine. Each point is the mean ± SEM from 9 to 10 rabbits.

IV. DISCUSSION

This study demonstrates that ricin administration causes changes in the responses of rabbit coronary arteries to vasoactive agents. Maximal contractions to histamine and serotonin were significantly increased. Maximal relaxations to NE and ACh were also increased, although not to a significant extent. The EC_{50}s of the coronary arteries to serotonin and histamine were increased, and that to NE was decreased which probably indicates heterologous sensitivity changes interior to the coronary artery effectors. These effects are in agreement with our previous findings on rabbit central ear arteries with NE, (Christiansen *et al.*, 1992), indicating that ricin may affect the coronary artery in the same way as it does the central ear artery.

Ricin administration increased the maximal contraction of coronary artery rings to serotonin and histamine. Contractions to both agents were increased by removal of the endothelium which is consistent with reports by Griffith *et al.* (1984). It has not yet been established whether this increase is mediated by evoked or spontaneous release of EDRF from the endothelium (Martin, 1988). Serotonin and histamine both stimulate G-protein coupled receptors and activate phospholipase C (PLC) which increases inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) and subsequently, cytosolic free Ca^{2+} concentrations (Hathaway *et al.*, 1991). Hsu *et al.* (1993) had some clues as to how ricin might act, in a study on calcium movements in the rabbit aorta, suggesting IP_3-induced calcium release from intracellular stores might be increased by ricin treatment. Whether the IP_3 level, PLC activity or receptor number on the cell membrane are affected is not clear. Furthermore, some changes in contractile elements such as phosphorylation of myosin light chain and the actomyosin ATPase activity could also affect muscle contraction.

Rabbit coronary arteries do not have endothelial alpha and beta adrenoceptors, at least no functional ones (Corr *et al.*, 1991). There are more beta adrenoceptors than alpha adrenoceptors on the smooth muscle of rabbit coronary arteries (Corr *et al.*, 1991). NE causes relaxation via beta adrenoceptors and endothelial removal does not increase the maximal response to NE. Ricin treatment increased the maximal relaxation to NE, although not significantly and decreased the EC_{50} to NE. Stimulation of beta adrenoceptors results in an increased intracellular cAMP concentration and activation of cyclic AMP-dependent protein kinase which lowers the cytosolic free Ca^{2+} concentration via the phosphorylation cascade. Because ACh relaxes rabbit coronary artery via an endothelium dependent mechanism, the lack of differences in ACh-induced relaxations between control and ricin administered rabbits indicates that ricin may not affect endothelium function in the coronary artery.

The regulation of coronary blood flow is complex. There are many factors involved in its regulation, including mechanical, neural and metabolic factors (Malindzak, 1980). The receptors investigated in our

experiments are only part of the coronary regulating system, but the results provide useful information for better understanding the mechanism of ricin intoxication. The normal serotonin concentration in rabbit plasma is about 0.1 μg/ml (5.6 X 10^{-7} M) (Waalkes, 1957a). Serotonin is available in blood from the platelets. In acute anaphylaxis, serotonin levels can rise to 1.6 μg/ml, but rapidly return to normal (Waalkes, 1957b). Platelets are usually activated by an immunoreaction or by hemostasis. The injection of minute amounts of ricin will not produce an anaphylaxis-like reaction. So, in the early stages after ricin injection plasma serotonin concentrations should be approximately normal at which the contractile tension of coronary artery rings from treated rabbits by serotonin was about 120 mg higher than that of control. The normal rabbit plasma histamine concentration is about 0.18 μg/ml (1.6 X 10^{-6} M) (Waalkes, 1957a), at which contraction of coronary artery from treated rabbits receiving ricin is decreased by 200-300 mg compared with those of control rabbits. Histamine is also available to the blood from basophils (Ganeling *et al.*, 1982). Histamine, like serotonin, is probably present in the plasma at about the normal level in early stage ricin intoxication. Also, adding in the increased sensitivity of the coronary artery to relaxation by NE, the sum of the effects of NE, histamine, and serotonin seems likely to be a relaxation of coronary arteries. This may partially contribute to the increased blood flow to coronary artery in the early stage of ricin intoxication in rabbits (Zhang *et al.*, 1992). Pathological studies of the rabbits that died from ricin and of the rabbits euthanized in the late stage of ricin intoxication or before dying from a high toxic dose of ricin demonstrated that the typical changes in heart, lungs and other organs are severe hemorrhage and necrosis (Christiansen *et al.*, 1991). These changes will markedly increase the amount of histamine and serotonin, at least locally (De Clerck *et al.*, 1984). Our results may be helpful for better understanding or predicting the hemodynamic changes in the heart in the late stage of ricin intoxication. Spasms of coronary artery and disseminated intravascular coagulation (DIC) possibly occur and may contribute to macro-and micro-circulatory collapse and death.

Severe myocardial hemorrhage (Christiansen *et al.*, 1991) and a drop in systolic and diastolic blood pressure after intravenous administration of a sublethal dose of ricin have been observed in the rabbit (Christiansen *et al.*, 1994a). Recently, we studied ricin's effects on cardiac function and electrophysiological properties in rabbit heart.

In our studies on the heart, we intended to determine whether ricin affects the contractility and electrophysiology of the isolated rabbit heart and isolated papillary muscle. We also evaluated the effects of ricin on alpha and beta adrenoceptor-mediated inotropic effects. Further, we tried to determine the mechanism of ricin's effects on the myocardial contraction and cardiac electrophysiology when there were alterations in the isolated heart and papillary muscle experiments.

No tendency toward altering LV, RV, or body weights by ricin

administration was observed in this study. In the papillary muscle experiments, ricin administration did not show significant positive inotropic or negative inotropic effects. Ricin administration did not alter time to the peak tension and contraction duration. No shortening or prolongation of action potential duration were observed. The V_{max} and action potential amplitude were not different between control and ricin administration groups. These findings indicated that ricin did not affect the intrinsic contractility of the papillary muscle and had no electrophysiological effects on the rabbit heart, in agreement with our previous findings *in vivo* (Christiansen *et al.*, 1994a), at least at 48 hours after the administration of a MLD of ricin.

The systolic pressure plotted against the balloon volume represents the capacity of the entire myocardium to generate systolic force (Weber *et al.*, 1976). In the isolated heart experiments, the systolic and developed pressure was decreased at a balloon volume of 1.3 to 1.5 ml. However, reduced force generation was not verified by a reduction in myocardial oxygen consumption, +dp/dt, and ventricular elastance. Contractility as assessed by changes in the maximal +dp/dt did not differ between the two groups. Elastance (Emax), introduced by Sagawa *et al.* (1977) as an index of myocardial contractility, had not deteriorated significantly in left ventricular systolic contractile function as demonstrated by the analysis of elastance in the two groups. There is also no evidence of decreased contractility from papillary muscle experiments to support a change in systolic and developed pressure. The reason for the decreased systolic and developed pressure, especially the decreased maximal LV developed pressure still needs to be determined.

Beta-adrenergic stimulation increases active tension and contractility and accelerates active relaxation (Aoyagi *et al.*, 1991). The first effect is mainly a consequence of an increased slow inward calcium current, while the second results from activation of the Ca^{2+}-ATPase of the sarcoplasmic reticulum via phosphorylation of phospholamban. Thus, it could be expected that, on an isolated heart, anything that can decrease the release of catecholamines as well as block or damage beta-adrenergic receptors would decrease the systolic pressure and relaxation velocity. In the isolated heart experiment, ricin did not alter the isoproterenol-induced increase in LV developed pressure. This is probably due to the fact that isoproterenol improves muscle compliance. The contractility reflected by +dp/dt, and muscle relaxation reflected by -dp/dt were not affected. Thus, overall the beta-adrenergic system was not altered by the MLD of ricin. This observation is consistent with the results of the binding studies and the studies on isolated papillary muscles. Similarly, the alpha-adrenergic system was not altered by ricin administration.

The major finding in the heart was that LV diastolic function changed after ricin administration. The isolated heart perfused according by the Langendorff procedure, is particularly suitable for studying ventricular compliance (Jalil *et al.*, 1989). The diastolic stiffness of the

chamber was increased by ricin administration. This reduction in diastolic compliance can be most easily explained by tissue edema or an increased amount of collagen. However, those measurements have not been made. A previous study has shown severe hemorrhage in the myocardium following ricin administration (Christiansen *et al.*, 1991). Hemorrhage disrupts cellular function, resulting in a redistribution of fluids and electrolytes. However, whether or not myocardial hemorrhage caused the diastolic dysfunction needs to be studied by electron microscopy to see whether there are cardiac changes in myocyte vacuolization, myofibrillar loss, and necrosis, or ultrastructural alterations including swelling of endothelial cells, interstitial hemorrhage, and edema.

The present investigation established that an MLD of ricin affects coronary artery and cardiac function, especially, diastolic function. This effect may not be directly on intrinsic myocardial contractility. The mechanism responsible for depressed cardiac function has not been thoroughly investigated. Ricin did not cause electrophysiological effects or alter the alpha and beta adrenergic-induced effects.

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VI. REFERENCES

Aoyagi, T., Momomura, S., Serizawa, T., Iizuka, M., Ohya, T., and Sugimoto, T. Alpha-adrenoceptor-mediated inotropism and beta-adrenoceptor-mediated inotropism in isolated rabbit ventricles: A comparison in mechanical effects and energetic efficiency. J. Cardiovascular Pharmacol. 17:647-655, 1991.

Araki, T. and Funatsu, G. The complete amino acid sequence of the B-chain of ricin E isolated from small-grain castor bean seeds. Ricin E is a gene recombination product of ricin D and Ricinus communis agglutinin. Biochim. Biophys. Acta 911:191-200, 1987.

Araki, T. and Funatsu, G. Revised amino acid sequence of the B-chain of ricin D due to loss of tryptophan in the cyanogen bromide cleavage. FEBS Letters 191:121-124, 1985.

Araki, T., Yoshioka, Y. and Funatsu, G. The complete amino acid sequence of the B-chain of the Ricinus communis agglutinin isolated from large-grain castor bean seeds. Biochim. Biophys. Acta 872:277-285, 1986.

Baenziger, J. U. and Fiete, D. Structural determinants of Ricinus Communis agglutinin and toxin specificity of oligosaccharides. J. Biol. Chem. 254:9795-9799, 1979.

Balint, G. A. Ricin: The toxic protein of castor oil seeds. Toxicol. 2:77-102, 1974.

Bruckner, R., Hackbarth, I., Meinertz, T., Schmelzle, B., and Scholz, H. The positive inotropic effect of phenylephrine in the presence of propranolol. Increase in time to peak force and in relaxation time without increase in c-AMP. Naunyn Schmiedebergs Arch Pharmacol 303:205-211, 1978.

Burnstock, G. Nonadrenergic innervation of blood vessels-some historical perspectives and future directions. In: Burnstock G, Griffiths S.G., eds. Nonadrenergic innervation of blood vessels. Vol. 1. Boca Raton, Florida: CRC Press, pp 1-14. 1988

Cawley, D.B. and Houston, L.L. Effect of sulfhydryl reagents and protease inhibitors on sodium dodecyl sulfate-heat induced dissociation of Ricinus Communis agglutinin. Biochim. Biophys. Acta 581:51-62, 1979.

Christiansen, V.J., Boatright, S.M., Hsu, C.-H. and Robinson, C.P. The effect of ricin on plasma norepinephrine concentrations and catecholamine metabolizing enzymes in the rabbit. The Toxicologist, 13:867, 1993a.

Christiansen, V.J., Patatanian, L., Boatright, S., Cardenas, J., Hsu, C.-H. and Robinson, C.P. The effects of ricin on norepinephrine (NE) efflux, uptake and content of rabbit aorta. FASEB J, 7:3960, 1993b.

Christiansen, V.J., Hsu, C.-H., Dormer, K.J. and Robinson, C.P. The cardiovascular effects of ricin in rabbits. Pharmacol. and Toxicol. In Press. 1994a

Christiansen, V.J., Hsu, C.-H., Kosanke, S.D. and Robinson, C.P. Histological and laboratory value abnormalities following ricin administration to rabbits. The Pharmacologist 33:210, 1991.

Christiansen, V. J., Hsu, C-H, and Robinson C. P. The effects of ricin on contractions and relaxations of rabbit arteries. The Pharmacologist, 34:83, 1992.

Christiansen, V.J., Hsu, C.-H. and Robinson, C.P. Effect of ricin on the sympathetic vascular neuroeffector system of the rabbit. J. Biochem. Toxicol. In Press. 1994a.

Corr, L., Burnstock, G., and Poole-Wilson, P. Responses of the rabbit coronary artery to acetylcholine and adrenoceptor agonists. Cardiovascular Res. 25:256-262, 1991.

Crompton, R. and Gall, D. Georgi Markov - Death in a pellet. Medico-Legal J. 48:51-62, 1980.

De Clerck, F, Van Neuton, J.M., and Reneman, R.S. Platelet-vessel wall interactions implication of 5-hydroxytryptamine. Agents Actions, 15, 612-626, 1984.

Derenzini, M., Benetti, E., Marinozzi, V. and Stirpe, F. Toxic effects of ricin: Studies on the pathogenesis of liver lesions. Virchows Arch. B 20:15-28, 1987.

Dixon, W,J. The up-and-down method for small samples. Am. Stat. Assoc. J. 60:967-984, 1965.

Ehrlich, P. The collected papers of Paul Ehrlich, Vol. 2. Pergamon: New York 1957, 21-44.

Eiklid, K., Olsnes, S. and Pihl, A. Entry of lethal doses of abrin, ricin and modeccin into the cytosol of HeLa cells. Exp. Cell Research 126:321-326, 1980.

Endo, Y., Mitsui, K., Motizuki M. and Tsurugi, K. The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. J. Biol. Chem. 262:5908-5912, 1987.

Endo, Y. and Tsurugi,K. RNA N-glycosidase activity of ricin A-chain. J. Biol. Chem. 262: 8128-8130, 1987.

Fodstad, O., Johannessen, J.V., Schjerven, L. and Pihl, A. Toxicity of abrin and ricin in mice and dogs. J. Toxicol. Environ. Health. 5:1073-1084, 1979.

Fodstad, O., Olsnes, S. and Pihl, A. Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice. Br. J. Cancer. 34:418-425, 1976.

Fodstad, O. and Pihl A. Effect of ricin and abrin on survival of L1210 leukemic mice and on leukemic and normal bone marrow cells. Int. J. Cancer 22:558-563, 1978.

Foxwell, B.M.J., Detre, S.I., Donovan, T.A. and Thorpe, P.E. The use of anti-ricin antibodies to protect mice intoxicated with ricin. Toxicology 34:79-88, 1985.

Funatsu, G., Kimura, M. and Funatsu, M. Primary structure of Ala chain of ricin D. Agric. Biol. Chem. 43:2221-2224, 1979.

Funatsu, G., Yoshitake, S. and Funatsu, M. Primary structure of Ile chain of ricin D. Agric. Biol. Chem. 42:501-503, 1978.

Funatsu, M., Funatsu, G., Ishiguro, M. and Hara, K. Properties of subunits of ricin D. Jap. J. Med. Sci. Biol. 26:30-32, 1973.

Funatsu, M., Funatsu, G., Ishiguro, S, Nanno, S. and Hara, K. Structure and toxic function of ricin. I. Purification procedures of ricin D. II. Subunit structure of ricin D. Proc. Jap. Acad. 47:713-723, 1971.

Ganellin, C.R., Parsons, M.E. (eds.), (1982): Pharmacology of Histamine Receptors. Wright/PSF, Bristol, Mass.

Godal, A., Fodstad, O., Ingebrigtsen, K. and Pihl, A. Pharmacological studies of ricin in mice and humans. Cancer Chem. Pharmacol. 13:157-163, 1984.

Griffith, T. M., Henderson, A. H., Huges Edwards, D., and Lewis, M. J. Isolated perfused rabbit coronary artery and aortic strip preparations: The role of endothelium-derived relaxant factor. J. Physiol. 351:13-24, 1984.

Griffiths, G., Leek, M. and Gee, D. The toxic plant proteins ricin and abrin induce apoptotic changes in mammalian lymphoid tissues and intestine. J. Pathol. 151:221-229, 1987.

Hansen, S., Petersen, O., Sandvig, K., Olsnes, S. and van Deurs, B. Internalized ricin and the plasma memebrane glycoprotein MAM-6 co-localize in the trans-golgi network of T47D human breast carcinoma cells. Exp. Cell Res. 185:373-386, 1989.

Harper, C.G., Gonatas, J.O., Mizutani, T. and Gonatas, N.K. Retrograde transport and effects of toxic ricin in the autonomic nervous system. Lab. Invest. 42: 396-404, 1980.

Hatakeyama, T., Ohba, H., Yamasaki, N. and Funatsu, G. Binding of saccharides to ricin E isolated from small castor beans. J. Biochem. 105:444-448, 1989.

Hatakeyama, T., Yamzasaki N. and Funatsu G. Further studies on the histidine residue located at the high affinity saccharide-binding site of ricin E. Agric. Biol. Chem. 54:1085-1086, 1990.

Hathaway, D.R., March, K.L., Lash, J. A., Adam, L.P., and Wilensky, R.L. Vascular smooth muscle. A review of the molecular basis of contractility. Circulation, 83:382-90, 1991.

Helke, C., Charlton, C. and Wiley, R. Suicide transport of ricin demonstrates the presence of substance P receptors on medullary somatic and autonomic motor neurons. Brain Res. 328:190-195, 1985.

Hsu, C-H, Zhang, L., Christiansen, V.J., and Robinson, C.P. Effects of in vivo ricin treatment on calcium flux in isolated aortas. The Pharmacologist, 35:155, 1993.

Jalil, J.E., Doering, C.W., Jnaicki, J.S., Pick, R., Shroff, S.G. and Weber, K.T. Fibrillar collagen and myocardial stiffness in the intact hypertrophied rat left ventricle. Circular Res. 64:1041-1050, 1989.

Keef, K.D., Pasco, J.S., and Eckman, D.M. Purinergic relaxation and hyperpolarization in guinea pig and rabbit coronary artery: role of the endothelium. J. Pharmacol. Exp. Ther. 260:592-600, 1991.

Ko, T. and Kaji, A. Effect of ricin on biosynthesis of myeloma protein (IgA) and general cellular proteins in MOPC-315 cells. Biochim. Biophys. Acta 414:155-160, 1975.

Koga, M., Ohtsu, M. and Funatsu, G. Cytotoxic, cell agglutinating, and syncytium forming effect of purified lectins from Ricinus Communis on cultured cells. Gann 70:585-591, 1979.

Larkin, J., Brown, M., Goldstein, J. and Anderson, R. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. Cell 33:273-285, 1983.

Leek, M., Griffiths, G. and Green, M. Intestinal pathology following intramuscular ricin poisoning. J. Pathol. 159:329-334, 1989.

Lin, J., Liu, K., Chen, C. and Tung, T. Effect of crystalline ricin on the biosynthesis of protein, RNA and DNA in experimental tumor cells. Cancer Res. 31:921-924, 1971.

Lin, J., Tseng, K., Chen, C., Lin, L. and Tung, T. Abrin and ricin: New anti-tumour substances. Nature 227:292-293, 1970.

Malindzak Jr., G.S. Differential segmental response characteristics of the coronary vasculature. In: The Coronary Artery (Kalsner, S., ed). Oxford University Press, New York, pp. 241-309, 1980.

Martin, W.: Basal Release of Endothelium-Derived Relaxing Factor. In, Relaxing and Contracting Factors. (Vanhoutte, P. M., ed). The Human Press Inc. Clifton, NJ. 1988.

Moya, M., Dautry-Varsat, A., Goud, B., Louvard, D. and Boquet, P. Inhibition of coated pit formation in Hep_2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. J. Cell Biol. 101:548-559, 1985.

Nanno, S., Ishiguro, M., Funatsu, G. and Funatsu, M. The mode of binding of carbohydrate in ricin D. Agric. Biol. Chem. 39:1651-1654, 1975.

Nicolson, G. Ultrastructural analysis of toxin binding and entry into mammalian cells. Nature 251:628-630, 1974.

Nicolson, G. and Blaustein, J. The interaction of Ricinus agglutinin with normal and tumor cell surfaces. Biochim. Biophys. Acta 266:543-547, 1972.

Nicolson G., Blaustein J. and Etzler M. Characterization of two plant lectins from Ricinus Communis and their quantitative interaction with a murine lymphoma. Biochemistry 13:196-204, 1974.

Nicolson, G.L., Lacorbiere, M. and Hunter, T.R. Mechanism of cell entry and toxicity of an affinity-purified lectin from Ricinus Communis and its differential effects on normal and virus-transformed fibroblasts. Cancer Res. 35:144-155, 1975.

Oda, T. and Funatsu, G. Cross-linking of the two constituent polypeptide chains of ricin D with N,N'-*O*-phenylenedimaleimide. Agric. Biol. Chem. 43:547-554, 1979.

Olsnes, S., Fernandez-Puentes, C., Carrasco, L. and Vazquez, D. Ribosome inactivation by the toxic lectins abrin and ricin. Eur. J. Biochem. 60:281-288, 1975.

Olsnes, S. and Pihl, A. Toxic lectins and related proteins. in: The Molecular Actions of Toxins and Viruses (eds Cohen, P. and van Heyningen, S.) pp. 52-105 Elsevier Biomedical, New York, 1982.

Olsnes, S. and Pihl, A. Treatment of abrin and ricin with beta-mercaptoethanol. Opposite effects on their toxicity in mice and their ability to inhibit protein synthesis. FEBS Lett. 28:48-50, 1972.

Olsnes, S. and Pihl, A. in: The Specificity and Action of Animal, Bacterial and Plant Toxins (Cuatrecasas, P., ed), Vol. 1 pp. 131-173, Chapman and Hall, London. 1976.

Olsnes, S., Refsnes, K. and Pihl, A. Mechanism of action of the toxic lectins abrin and ricin. Nature 249:627-639, 1974.

Olsnes, S. and Sandvig, K. Entry of toxic proteins into cells. In: Receptors and Recognition, Series B, vol. 15 (Cuatrecasas, P., and Roth, T.F. eds.) pp. 189-236, Chapman and Hall Ltd., London, 1983.

Olsnes, S., Sandvig, K., Refsnes, K. and Pihl A. Rates of different steps involved in the inhibition of protein synthesis by the toxic lectins abrin and ricin. J. Biol. Chem. 251:3985-3992, 1976.

Refsnes, K., Olsnes, S. and Pihl, A. On the toxic proteins abrin and ricin. J. Biol. Chem. 249:3557-3562, 1971.

Sagawa, K., Suga, H., Shoukas, A.A. and Bakalar, K.M. End-systolic pressure/volume ratio: A new index of ventricular contractility. Amer. J. Cardiol, 40:748-753, 1977.

Sandvig, K. and Olsnes S. Effect of temperature on the uptake, excretion and degradation of abrin and ricin by HeLa cells. Exp. Cell Res. 121:15-25, 1979,

Sandvig, K. and Olsnes S. Entry of the toxic proteins abrin, modeccin, ricin and diphtheria toxin into cells. I. Requirement for calcium, and II. Effect of pH, metabolic inhibitors and ionophores and evidence for toxin penetration from endocytic vesicles. J. Biol. Chem. 257:7495-7513, 1982.

Sandvig, K., Olsnes, S. and Pihl, A. Binding, uptake and degradation of the toxic proteins abrin and ricin by toxin-resistant cell variants. Eur. J. Biochem. 82:13-23, 1978.

Sandvig, K. Tonnessen, T. and Olsnes, S. Ability of inhibitors of glycosylation and protein synthesis to sensitize cells to abrin, ricin, Shigella toxin and Pseudomonas toxin. Cancer Res. 46:6418-6422, 1986.

Schumann, H.J., Endoh, M., and Wagner, J. Positive inotropic effects of phenylephrine in the isolated rabbit papillary muscle mediated both by alpha- and beta-adrenoceptors. Naunyn Schmiedebergs Arch. Pharmacol. 284:133-148, 1974.

Silverstein, S. C., Steinman, R. M. and Cohn, Z. A. Endocytosis. Annu. Rev. Biochem. 46: 669-722, 1977.

Simmons, B., Stahl, P. and Russell, J. Mannose receptor-mediated uptake of ricin toxin and ricin A chain by macrophages. J. Biol. Chem. 261:7912-7920, 1986.

Skilleter, D. N. and Foxwell, B.M.J. Selective uptake of ricin A-chain by hepatic non-parenchymal cells in vitro. FEBS 196: 344-348, 1986.

van Deurs, B., Sandvig, K., Petersen, O. and Olsnes, S. Endocytosis and intracellular sorting of ricin. In: Trafficking of bacterial toxins. Saelinger, C. ed. CRC Press pp. 91-119, 1990.

van Deurs, B., Tønnenssen, T., Petersen, O., Sandvig, K. and Olsnes, S. Routing of internalized ricin and ricin conjugates to the Golgi complex. J. Cell Biol. 102:37-47, 1986.

Villafranca, J.E. and Robertus, J. D. Ricin B chain is a product of gene duplication. J. Biol. Chem. 256:554-556, 1981.

Waalkes, T.P., Weissback, H., Boricevich, J., and Undenfriend, S. Serotonin and histamine release during anaphylaxis in the rabbit. J. Clin. Invest., 36:1115-20, 1957a.

Waalkes, T.P., Weissback, H., Boricevich, J., and Undefriend, S. Further studies on release of serotonin and histamine during anaphylaxis in the rabbit. Proc. Soc. Exp. Biol. Med., 95:479-82, 1957b.

Weber, K.T., Janicki, J.S., Reeves, R.C. and Hefner, L.L. Factors influencing left ventricular shortening in isolated canine heart. Amer. J. Physiol. 230:419-426, 1976.

Wiley, R.G., Blessing, W.W. and Reis, D.J. Suicide transport-destruction of neurons by retrograde transport of ricin, abrin and modeccin. Science 216:889-890, 1982.

Wurtman, R.J. and Axelrod, J. A sensitive and specific assay for the estimation of monoamine oxidase. Biochem. Pharmacol. 12:1439-1440, 1963.

Yoshitake, S., Funatsu, G. and Funatsu, M. Isolation and sequences of peptic peptides, and the complete sequence of Ile chain of ricin D. Agric. Biol. Chem. 42:1267-1274, 1978.

Zhang, L, Mills, S., Basmadjian, G.P., and Robinson, C.P. Effects of ricin on blood flow in the rabbit. The Pharmacologist, 34: 156, 1992.